



Università degli Studi di Milano
Dipartimento di Scienze Agrarie e Ambientali – Di.S.A.A.
Dottorato di Ricerca in Biologia Vegetale e Produttività della Pianta
Coltivata - Ciclo XXVIII

PHYSIOLOGICAL AND ECOLOGICAL DRIVERS AND AGRONOMICAL CONSEQUENCES OF THE OZONE-LIKE SYNDROME IN WHEAT

Doctoral Thesis



Tutor: Prof. Franco FAORO
Co-tutors: Prof. Giacomo GEROSA
Dr. Riccardo MARZUOLI

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Matricola: *R10063*

Accademic Year 2014-2015



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Dedication

I dedicate this work:

To my father and my mother.

*To my dear brothers **Igr. Eric MBAYO Ilunga**,...*

*To my wife **Nathalie MANDE** and my four lovely children, **Agnès B. ILUNGA**,
Baudouin ILUNGA. K, **Enock Y. ILUNGA** and **Robert M. ILUNGA**.*

To my uncles and aunts.

And

To all my friends.

Robert MONGA ILUNGA DIKOSHI

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Robert MONGA ILUNGA DIKOSHI

Summary

Tropospheric ozone (O₃) is known to adversely affect the productivity of a wide range of crops including wheat. However different species, can exhibit different responses to ozone exposure. Since the *Triticum* genus (wheat) is one of the most cultivated and consumed cereal on the global scale and also considered an O₃-sensitive crop, research on its protection against ozone damages can contribute to the improvement of its productivity and thus the worldwide food security.

The first part of this research was focused on a varietal screening experiment carried out in 2013 to assess the ozone sensitivity of 3 Italian and 2 Spanish cultivars of durum wheat (*Triticum durum*), applying two different levels of ozone (50% increment and 50% decrement of the ambient ozone concentration) to plants grown in Open-Top Chambers. The durum wheat sensitivity to ozone was based on the assessment of leaf visible injuries, histochemical observations, physiological parameters, yield and yield quality analysis. Two Italian cultivars (Colombo and Sculptur) resulted more sensitive to ozone than the others, according to the physiological parameters tested and to the grain yield and quality analysis. However, they also showed different levels of leaf visible and microscopic injuries. The flag-leaves of cv Colombo resulted clearly more damaged by ozone exposure than the flag-leaves of cv Sculptur at both visible and microscopic levels.

The second part of the research tried to analyze to a deeper extent the response to ozone exposure of the two most sensitive cultivars selected from the previous experiment. A second experiment was performed in 2014 using four ozone levels: -5% and -50% of ambient ozone concentration in non-filtered and charcoal-filtered OTCs, respectively; +30 and +60% of ambient ozone concentration in ozone-enriched OTCs (OZ+ and OZ++ OTC).

In order to test the effectiveness of an antitranspirant compound in protecting durum wheat from ozone oxidative stress, a chitosan solution was weekly applied as leaf spraying during the growing season in 2014. The chitosan treatments were applied at 3 levels: tap-water (CTRL, no chitosan), 40kDa chitosan solution (CHI40) and 300kDa chitosan solution (CHI300). Both durum wheat cultivars confirmed their sensitivity to ozone as observed in the previous experiment. Grain yield losses observed in ozonated treatments were related to a decrease of stomatal conductance that is due to damages to the Rubisco and Calvin cycle. No protective effect due to chitosan treatments was observed in both

cultivars. However, chitosan improved slightly the grain yield and the aboveground biomass production in plants grown in charcoal-filtered and non-filtered OTCs. Biomass data were also used for the definition of dose-effect relationships based on the ozone exposure (AOT40), the phytotoxic ozone dose (POD₆) and the yield losses. The grain yield losses were plotted against AOT40 and POD₆ in order to test the linear regression of these two indices. Each increase of AOT40 3000 ppb.h caused a grain yield loss of about 1.8%, while for the POD₆, an increase of 1mmol O₃ m⁻² caused 1.3% reduction. Both AOT40 and POD₆ resulted appropriated for assessment of durum wheat yield losses. However, the dose-effect relationship based on POD₆ showed a better fit compared to the AOT40.

During the 2014 experiment an important part of the research regarded the ultrastructural analysis of ozone-like symptoms on flag-leaves carried out by transmission electron microscopy (TEM), and the assessment of the levels of some antioxidant molecules (ascorbate and glutathione) involved in the ozone-detoxifying process, to understand the mechanisms underlying the different ozone sensitivity of Colombo and Sculptur in terms of visible and microscopic symptoms. Results from TEM demonstrated that visible symptoms in Colombo are due to the presence of damaged stomata and plasmolyzed mesophyll cells around the sub-stomatal cavity. On other hand, no damage on stomata, mesophyll cells and chloroplasts were observed in Sculptur cultivar explaining the absence of the visible symptoms. In general Sculptur showed higher levels of ascorbate content than Colombo, suggesting a higher capacity ascorbate biosynthesis. No significant difference in ascorbate content was found between plants exposed and not exposed to elevated ozone. The total and the oxidized glutathione content increased in the Colombo cultivar grown in elevated ozone conditions indicating that plant ability to maintain glutathione in the reduced form was decreased by the ozone stress.

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Chapter 1.

General introduction

Tropospheric ozone (O_3) is a secondary atmospheric pollutant generated from photochemical reactions involving the oxidation of volatile organic compounds (VOC) and carbon monoxide (CO) in the presence of nitrogen oxides (NO_x), high temperatures and high solar radiation (U.S. EPA, 2004; Volz-Thomas et al., 2002; Liu *et al.*, 2007). High levels of ozone during the spring-summer periods can affect human health by irritating the eyes, mucous membranes and causing negative effects to the respiratory system. Moreover this pollutant can have detrimental impacts on natural vegetation and crop yield (Fuhrer and Achermann, 1994; IPCC, 2007; Shi *et al.*, 2009) with a wide range of significant damages in plants at biological, physiological and ecological level (Yu *et al.*, 2001; Langkulsen, 2006; Rodriguez *et al.*, 2007; Simon and Charpin, 2011; Yu *et al.*, 2009; De Andrés *et al.*, 2012).

The past three decades have been characterized by an increase of O_3 concentration of 0.5-2% per year in the Northern hemisphere, while the current background O_3 concentration in this world area is in the range of approximately 23-34 nmol mol⁻¹ (Vingarzan, 2004; Singh *et al.*, 2013) and is expected to increase by 40-70% by the end of 21st century (Zeng *et al.*, 2008). Furthermore, climate change scenarios foresee an increase of global temperatures of about 1.4-5.8°C, coupled with changes in precipitation and increased frequency of extreme weather events (IPCC, 2001) suggesting a potential further increase of ozone levels due to favourable conditions.

Ozone enters the plant leaves exclusively through open stomata during the normal gas-exchange process. Once inside the plant, it produces several reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl (OH^\bullet) and hydroperoxyl (HO_2) radicals (Overmyer *et al.*, 2009; Turcsányi *et al.*, 2000; Vahisalu et., 2010). Reactive oxygen species are able to initiate membrane lipid peroxidation, chlorophyll bleaching, protein oxidation, damage to nucleic acids and destruction of cell membranes and chloroplasts (Eltner *et al.*, 1994; Karberg *et al.*, 2005; Vaultier and Jolivet, 2015). In response to these stressors, plants can activate a number of antioxidative stress-related defense mechanisms (Smirnoff., 1993; Kangasjärvi *et al.*, 1994). Important antioxidants of plants against ozone are: ascorbate, phenolics, α -tocopherol, glutathione, carotenoids and enzymes such as superoxide dismutases, catalases and several peroxidases (Temmerman *et al.*, 2002).

The main effects of high ozone concentrations on vegetation at physiological level are related to a decreasing photosynthetic capacity resulting from reduced stomatal

conductance, light-saturated photosynthesis (A_{sat}), maximum activity of Rubisco (V_{cmax}) and maximum electron transport rate (J_{max}), and causing an alteration of the assimilate partitioning (Cao *et al.*, 2009; Li *et al.*, 2015; Saitanis *et al.*, 2015; Nouchi *et al.*, 1995). Additionally, ozone exposure can cause chlorotic and necrotic lesions on leaves of sensitive plants species (Benton *et al.*, 2000; Chaudhary and Agrawa, 2015), although photosynthesis and growth can be inhibited even in absence of leaf visible symptoms.

At individual level, ozone exposure can induce significant losses of crop yield and crop quality (Sarkar and Agrawal, 2010; Gerosa *et al.*, 2009). For example, wheat yield losses due to ambient ozone concentrations were estimated at about 20-27% in the Mediterranean region (Fumagalli *et al.*, 2001) and at about 6-17% in the central Italy (Fagnano *et al.*, 2009). High ozone concentration is also known to influence negatively the soil-root respiration (Chen *et al.*, 2010) and the soil microbial biomass and community structure (Manninen *et al.*, 2010; Li *et al.*, 2012; Bao *et al.*, 2015) by altering plants' belowground processes, and hence the carbon (C) and nitrogen (N) cycles in soils (Larson *et al.*, 2002). The study of Feng *et al.* (2015) on rice cultivars suggests the contrasting responses of the bacterial community.

Ozone has been reported to have adverse effects on the ecosystem productivity through influencing leaf photosynthesis, respiration, stomatal conductance, carbon allocation, litter decomposition, water cycling and community properties such as species diversity, functional types and dominant vegetation types (Neufeld *et al.*, 2006; Matyssek and Sandermann, 2003; Ren *et al.*, 2007).

It should be noted that in Mediterranean conditions, other abiotic stresses, such as drought and water salinity may influence the crops sensitivity to ozone by limiting pollutant entree in plant through stomata (Katerji *et al.*, 2008; Maggio and Fagnano, 2010; Gerosa *et al.*, 2014). Italy is the second world producer of durum wheat after Canada. According to International Grains council (IGC), Canada produced 6.5 million tons of durum wheat in 2013/2014 season and Italy comes right after Canada with about 4 million tons. Italian durum wheat production represents half of the whole EU (European Union) production (Eurostat, 2014). Furthermore, Italy is among the leading producers and exporters of pasta in the world with 3.3 million tons including 1.7 exported. Based on the foregoing, Italy plays a crucial role in the research on the improvement of the productivity of this species (Bozzini *et al.*, 1998; Motzo *et al.*, 2007). The agricultural yield increase is primarily related to the plant breeding (De Vita

et al., 2007; Araus *et al.*, 2002; Motzo *et al.*., 2007) and photosynthetic efficiency is among the most important characters that can be implemented even if in the case of wheat, the photosynthetic efficiency is low and represents only a quarter of that of corn (Gonzales *et al.*, 2003). Since the photosynthetic efficiency can be enhanced with an increase of the stomatal conductance (and hence increasing the stomatal fluxes), it has been observed that the most productive cultivars of wheat produced in the last decade were also the most exposed to oxidative stresses due to air pollutants (Biswas *et al.*, 2008b).

It is therefore necessary that geneticists select varieties of durum wheat, which will be more tolerant to oxidative stress, for example, increasing the pool of antioxidant of leaf tissues, and that improve the productivity. Unfortunately, the selection of these genotypes is not easy and so far the tolerant varieties in that field were asymptomatic showing themselves always significantly less productive. In any case, the genetic improvement requires medium-long times, then all the agronomical approaches designed to mitigate the oxidative stress and possibly to make the plants more resistant to pathogens they are greatly desired in the immediate, also in view of the above reported, or that the increase of the production of even a few percentage points would have a very significant global effect. This is what emerged in the recent World Symposium named "Wheat for the Future - The progress of research on wheat for global food security" organized by the CNR (National Research Council, Consiglio Nazionale delle Ricerche) within the framework of EXPO 2015, where they discussed strategies for increasing productivity and the quality of durum wheat for the next decades in function of global climate change. If in fact the temperature by 2050 would increase as little as two degrees, the production of durum wheat would fall by about 20% (Amell *et al.*, 2013), posing a serious food safety problems if not treated by all means immediately.

Objectives of the thesis

The main objective of this study is to contribute to bridge the gap of knowledge on the effects of O₃ on *Triticum durum* (chapter 2) in the Mediterranean conditions.

The specific aims of the research activity were:

- i) to test whether different cultivars of *Triticum durum* show a different tolerance/sensitivity to tropospheric O₃ in terms of diffusion of leaf injuries, plant growth and grain yield;
- ii) to characterize the response to O₃ of two durum wheat cultivars (Colombo and Sculptur) that were found particularly sensitive to O₃ in the varietal screening, by investigating the possible role of antioxidants pool, photosynthesis efficiency and stomatal conductance in the variation of tolerance/sensitivity to O₃ between the two cultivars;
- iii) to assess the potential role of chitosan (an anti-transpirant product) in protecting wheat plants from ozone damages;
- iv) to provide new information for improving risk assessments of the impact of O₃ on durum wheat production in Mediterranean region.

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Chapter 2

A varietal screening of ozone sensitivity in Mediterranean durum wheat (*Triticum durum*, Desf.)

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Abstract

This study investigated the ozone (O₃) sensitivity of five cultivars of durum wheat (*Triticum durum*) grown in Open-Top Chambers (OTC) during the 2013 growing season. Two levels of ozone were applied during daylight hours: +50% and -50% of ambient ozone concentration respectively in O₃-enriched OTC and charcoal-filtered OTC. Results suggest that the significant differences observed in agronomic parameters, were more cultivar-dependent rather than ozone-dependent. Two cultivars showed a reduction of grain yield due to ozone between 10% and 16% , however this decrease was significant only for one of them (Sculptur). On the contrary, a very slight increase of grain yield was reported for the other cultivars. Stomatal conductance was significantly reduced by ozone fumigation, from -18% in the morning to -33% in the afternoon measuring cycle respectively. No significant effects on chlorophyll fluorescence were found, nor correlation was observed between ozone-like symptoms severity (leaf chlorotic/necrotic spots) and yield reduction, suggesting that these parameters cannot be indicative of ozone sensitivity/tolerance. These results may be useful for the selection of durum wheat genotypes more adapted for the cultivation in geographical areas where tropospheric ozone is particularly high, but also for the future definition of consistent dose-response relationships to be used in the ozone risk assessment evaluation for the Mediterranean countries.

Key words: durum wheat; ozone; grain yield; stomatal conductance.

2.1 Introduction

Tropospheric ozone (O₃) is widely recognized as a phytotoxic atmospheric pollutant. Background O₃ concentrations over the last decades have been, and are still, increasing in Europe although peak concentrations are decreasing (Vingarzan, 2004; Sicard *et al.*, 2013; Paoletti *et al.*, 2014). In the Mediterranean area of Europe, ozone concentrations frequently exceed limit values established for the protection of natural vegetation and crops (Paoletti and Manning, 2007; EEA, 2013). Furthermore, climate change projections will cause a rise in temperature and likely even higher ozone background concentrations. Models have predicted a 3-5°C increase in temperature (IPCC, 2007) and changes in tropospheric ozone from -12% to +62% was calculated for the 21st century (Vingarzan *et al.*, 2004; Dentener *et al.*, 2006; IPCC, 2001).

Raising phytotoxic O₃ concentrations in rural areas of Europe and other regions of the world are a cause of concern. Agricultural production in general, and wheat cultivation in particular, is affected by tropospheric ozone in the developed and industrialized regions (Emberson *et al.*, 2009; Kaliakatsou *et al.*, 2010; Feng and Kobayashi, 2009; Mills and Harmens, 2011; Avnery *et al.*, 2011). Recognized as a significant stressor, ozone can lead to a functional unbalance of plants by altering the gas exchange at leaf level as a consequence of direct stomata impairment caused by the Reactive Oxygen Species (ROS) generated from this pollutant (Picchi *et al.*, 2010; Faoro and Iriti, 2005). Long-term exposure of plants to ozone could impact negatively their hormonal activities and progressively constrain plant defense arsenal, thus causing biochemical and physiological alterations in the whole plant (Diara *et al.*, 2005; Morgan and Drew, 1997; Wilkinson and Davies, 2010). A number of experiments reported reductions of stomatal conductance (Paoletti and Grulke, 2010; Grulke *et al.*, 2007), plant growth, plant productivity (Akhtar *et al.*, 2010b; Feng *et al.*, 2008; Booker *et al.*, 2009; Fangmeier *et al.*, 1994), yield quality and economic losses (Vlachokostas *et al.*, 2010; Holland *et al.*, 2002; Van Dingenen *et al.*, 2009) on crops.

Risk assessments of ozone effects on agricultural production performed by different international organisms like the Convention on Long-Range Transboundary Air Pollution (CLRTAP/UNECE) or the European Environment Agency are based either on dose-response or exposure-response relationships derived from published data on the response of common wheat (*Triticum aestivum*), a sensitive crop widely grown in Central and Northern Europe (Pleijel *et al.*, 2007; Fuhrer *et al.*, 1997). Other studies on the impacts of tropospheric ozone on agricultural production also rely on the response of common wheat (Avnery *et al.*, 2011; Feng *et al.*, 2012). Indeed common wheat is one of the most sensitive cereal crops to O₃ exposure (Pleijel *et al.*, 2006; Sarkar and Agrawal, 2010). Results of meta-analysis performed on *T. aestivum* by Feng *et al.* (2008) reported a decrease of 29% of grain yield with high levels of ozone concentration (31-200 ppb) and of 18% when ambient ozone concentration was between 31 and 59 ppb. A grain yield decrease between 13 and 21% for *Triticum aestivum* was also found by Rai *et al.* (2007), Pleijel *et al.* (1991) and Ollerenshaw and Lyons (1999) in other field and OTCs studies.

Less information is available about the ozone sensitivity of other wheat species. Durum wheat (*Triticum durum*) is one of the most adapted cereals to the Mediterranean environmental conditions and is able to maintain high gas exchange rate under high

temperatures and VPD, with a productivity up to 6 tons/hectare in rain-fed cultivation (González-Fernández *et al.*, 2013; Moragues *et al.*, 2006; Nachit and Elouafi, 2004). World's durum wheat production is estimated at about 5% of the global wheat production, 35% out of it is produced in the North Africa and West Asia, 30% in the EU and 25% in North America (Dixon *et al.*, 2009). Italy is the first EU durum wheat producer with a mean production of 4.22 million of tons on a mean area of 1.39 million hectares cultivated in the last ten years (2004-2013). Spain is also among the biggest durum wheat producers in Europe with a mean production of 1.24 million of tons on a mean cultivated area of 0.558 million of hectares in the same period. Italy and Spain productions together are estimated at about 60% of all the EU production (EUROSTAT, 2014). Durum wheat covers roughly 50% of the surface cultivated with wheat in European Mediterranean countries (Abad *et al.*, 2004). Despite its importance, there is limited evidence of the sensitivity to ozone of durum wheat growing under Mediterranean conditions. The few studies available evidence that *Triticum durum* can be more O₃-tolerant than common wheat (Gerosa *et al.*, 2014; Biswas *et al.*, 2008a; Herbiner *et al.*, 2002). New information on the sensitivity of crops to ozone is very much needed in order to correctly evaluate the negative effects of ozone on staple crops like wheat in the Mediterranean area.

Many authors agree that the response of wheat and other crops to O₃ might be genotype-dependent and that O₃ sensitivity can vary as much as 30% across cultivars (Zhu *et al.*, 2011; Morgan *et al.*, 2006; Pleijel *et al.*, 2006; Tiwari *et al.*, 2005; Danielsson *et al.*, 2003; Fiscus *et al.*, 2005; Mills *et al.*, 2007; Biswas *et al.*, 2008b; González-Fernández *et al.*, 2010, 2014). This variability is not currently considered in the evaluation of O₃ effects on agricultural productivity. Furthermore, it offers a considerable scope for adaptation strategies at the farm level to raising O₃ concentrations projected for the coming decades.

The aim of the present study was to test the tolerance/sensitivity to tropospheric O₃ of five cultivars of *Triticum durum* in terms of leaf injuries, plant growth and grain yield. This information will be helpful for improving risk assessments of O₃ impacts on agricultural production based on dose-response relationships, and for identifying O₃-tolerant cultivars for future use in the Mediterranean region where elevated ambient O₃ concentration often threatens wheat cultivation.

2.2 Materials and Methods

2.2.1 Experimental design and plant material

The experiment was performed between March and July 2013 in the Open-Top Chambers (OTC) experimental site of C.R.I.N.E.S (Research Center on Air Pollution and Ecosystems), which is located inside the Regional Center for the Enhancement of Forest Biodiversity (formerly Forest Nursery) of the Lombardy Region at Curno, Bergamo (Lat. 45° 41' 17'' N, Long. 9° 36' 40'' E, elev. 242 m a.s.l.) in Northern Italy.

Seeds of 3 Italian cultivars (Sculptur, Colombo and Pharaon) and 2 Spanish cultivars (Gallareta and Vitron) of durum wheat were sown on 9 March 2013 in 60 pots (12 pots for each cultivar, 25 cm diameter, 10L volume) filled with a standard commercial soil (Koro Excell universal) with C/N ratio 31 and pH between 5.5 and 6.5. Each pot contained three plants, and a total of 12 pots of each cultivar were subdivided and located in 4 OTCs (3 pots per OTC) on 2 April 2013. Automatic irrigation was performed starting from June to maintain the soil water content of the pots close to field capacity.

A split-plot experimental design was applied, with “ozone” treatment as the main factor at two levels (CF-OTC, EN-OTC) and with “cultivars” as nested factor at 5 levels.

Two OTCs were equipped with activated charcoal filters (Charcoal-Filtered OTC, CF-OTC). The air filtration system assured an abatement of about 50% of ambient air ozone concentration inside the OTC. The other two OTCs (OZ++OTC) were fed with O₃-enriched air (O₃-enriched OTC, OZ++OTC). The ozone enrichment system consisted of an oxygen generator (OG-20, OGSi, N.Y. USA) coupled with an ozone generator (OGF 500, Pilodist GmbH, Germany) and a set of PTFE pipes to bring generated ozone directly to the OZ++OTCs. The ozone was injected into the OTC's air ventilation system and diluted with the ambient air prior to enter the OTC. A control system maintained the ozone concentrations inside OZ++OTCs about 50% higher than the ambient air concentrations for 8 hours a day (9:00-17:00), starting from 6 May (just before the emergence of flag leaves) until 30 June, for a total of 56 days of fumigation.

Ozone concentrations, within each OTC, were continuously monitored by an automatic ozone analyzer (model 1108 RS, Dasibi Italia s.r.l., I) via a solenoid valve switching system, which was managed by a dedicated PC equipped with a NI-DAQ 6.9 I/O board (National Instruments, Austin, TX) and a Labview 6.1 program devised specifically for this experimentation. The accumulated O₃ exposure over a threshold of 40 ppb (AOT40) is

used to report the exposure of plants per O₃ treatment. AOT40 is calculated as the sum of ozone concentrations over 40 ppb during daylight hours (Fuhrer *et al.*, 1997).

The main agrometeorological variables were monitored with a thermo-hygrometer sensor (50Y Campbell Sci.,USA), a PAR sensor (LI-190 LiCOR, USA), an anemometer (WSS1 Environmental Measurements Lim.,UK) and a rain gauge (52202 Young, USA) that were installed at the center of one of the OTCs at 100 cm of height.

A CR10X datalogger with AM 16/32 multiplexing system (Campbell Scientific Inc., USA) was used in order to automatically acquire data from all of the field sensors.

2.2.2 Crop yield

Plant harvest was carried out 129 days after sowing (15th July). A total of 18 plants for each cultivar and ozone treatment were collected and measured to investigate the main crop yield parameters and plant biomass. The height of the plants was measured at the end of the growing season just before the harvest. Then, the aboveground biomass was divided into straw and spikes. Straws and spikes were dried in an oven at 80° C for 48 hours and weighed to obtain the dry weight. Spikes were shelled and the seeds were weighed to assess the grain yield and the harvest index of the crops (defined as the ratio between grain yield and total aboveground dry biomass).

2.2.3 Stomatal Conductance

Measurements of the stomatal conductance (g_s) to water were performed 4 times during the season on the adaxial surface of flag leaves. Three plants randomly chosen for each cultivar and OTC were measured with a dynamic diffusion portable porometer (AP4 Delta-T Devices, UK). The 4 measurements campaign began just before the anthesis and were carried out on the following dates: on 27 May, 7, 13 and 16 June. During each measurement day, g_s was recorded in three different times of the day, denominated as “morning” (from 8:30 to 10:00), “midday” (12:30-14:00), and “afternoon” (16.30-18:00) cycles. Before each measurement cycle, the porometer was calibrated according to the relative humidity values detected inside the OTCs.

2.2.4 Chlorophyll a fluorescence.

Fast induction kinetic of chlorophyll *a* fluorescence of the Photosystems II (PSII) was measured using a portable Handy-PEA (Plant Efficiency Analyzer) fluorimeter (Hansatech Instruments, Norfolk, UK). Measurements were performed after 30 minutes of dark adaptation, on the adaxial surface of the flag leaf of one plant for each pot of the different

cultivars in all of the OTCs, and replicated twice during the growing season, on 29 May and 7 June.

Data were elaborated with PeaPlus (v. 1.10) software in order to calculate the Performance Index (PI_{abs}) of the plants, which summarizes the photosystems' capacity for energy conservation of photons absorbed through the electron transport chain and for the reduction of the electron acceptors in the intersystem between PSII and PSI (Strasser *et al.*, 1999).

2.2.5 Leaf visible injuries and histochemical observations

Plants in all of the OTCs were monitored weekly from the beginning of the ozone treatments looking for the onset of chlorosis and necrosis symptoms typical of ozone injury on durum wheat (Picchi *et al.*, 2010). For histochemical observations three samples of flag leaves for each cultivar were collected from the CF-OTCs and from the EN-OTCs on 15 May, when visible symptoms had already started to appear. Samples (2x2 cm) were excised from the flag leaves and infiltrated with 3,3'-diaminobenzidine (DAB)-HCl to detect possible H₂O₂ accumulation sites, due to ozone-induced oxidative stress. The detailed protocols can be found in Faoro and Iriti (2005).

2.2.6 Statistical analysis

The statistical significance of the differences observed between plants of the different ozone treatments, regardless of the cultivar, was assessed with an analysis of variance (ANOVA) by considering the OTC as the statistical unit within a split-plot experimental design. Ozone was set as the main (fixed) factor while the cultivar was set as a nested factor within each ozone level. The normal distribution of the data of each parameter within each treatment group was verified by the Shapiro-Wilk W Test and by normal probability plots. The assumption of the homogeneity of the variances was verified for each parameter by the Levene's test.

A contrast analysis with a-priori planned comparisons was used to test the significance of the response of each single cultivar to ozone.

The statistical analysis was performed using STATISTICA 8.0 software (Tulsa, USA) in order to assess the effects of O₃ treatment on each measured parameter for all cultivars (global effect) and even the single cultivar and/or the interaction between ozone and cultivar or interaction ozone-cultivar-cycle of measurement in the case of stomatal conductance. The one-way analysis of variance (ANOVA) was used to test the sensitivity

of the five cultivars to O₃ exposure in terms of growth parameters, stomatal conductance, chlorophyll fluorescence and yield parameters. Difference between ozone treatments was considered significant at $p \leq 0.05$ (*) and at $p \leq 0.01$ (**) and highly significant at $p \leq 0.001$ (***), and not significant at $p > 0.05$ (ns).

2.3 Results

2.3.1 Climatic variables and ozone exposure

Table 2. 1- Monthly average of meteorological conditions, ozone exposure (AOT40) in the different ozone treatments,. Temp.= temperature; RH= relative humidity; CF-OTC= Charcoal-Filtered OTC; OZ++OTC= O₃-enriched OTC; AOT40= Accumulated Ozone over a Threshold of 40ppb.

	Average temperature °C	Average RH %	Total rain* mm	Rainy days n°	AA AOT40 ppb.h	CF-OTC AOT40 ppb.h	OZ++OTC AOT40 ppb.h
March	7	77	131	13	0	0	0
April	14	77	141	16	510	409	510
May	17	73	276	22	1109	3	6'935
June	23	65	42 (+115)	4	4993	146	9'637
July**	26	60	16 (+75)	3	4064	268	4'039

* In brackets the water received with automatic irrigation system.

** Only the period 1-15 July was considered

Table 2.1 reports the main climatic conditions (T, RH and rain) monitored inside the OTCs and the ozone exposure (AOT40) calculated during each month of the experiment.

Regarding the meteorological variables, it is worth remarking that the so called “chamber effect” is responsible for higher air temperatures (about 2-3°C) and lower air relative humidity (about -10%) inside the OTCs than in the ambient air.

The 2013 growing season was characterized by unusual cold spring, with low temperatures and frequent precipitations during April and May, when 16 and 22 days of rain were recorded respectively (**Table 2.1**); this amount of precipitation was significantly higher than the thirty-years mean recorded in the same location and period.

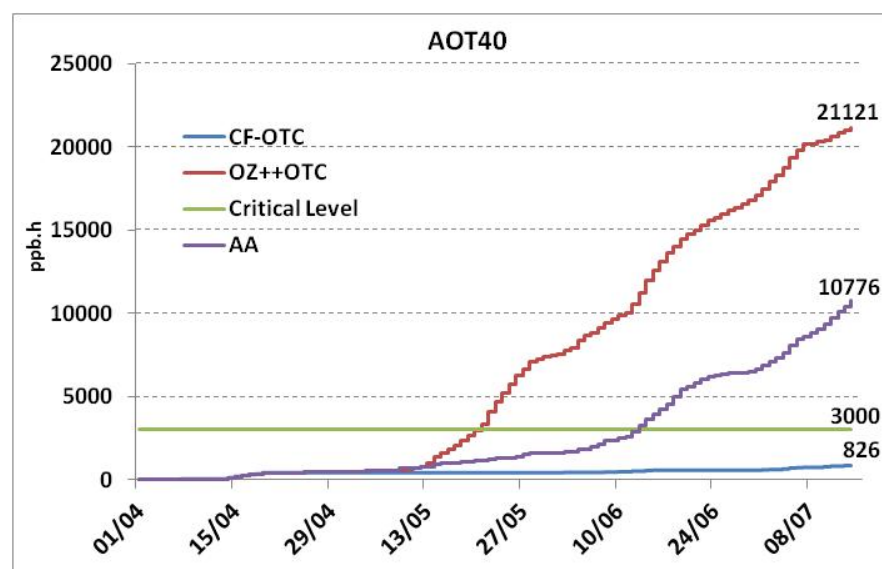


Figure 2. 1- Seasonal evolution of AOT40 index in the two different ozone treatments (CF-OTC and EN-OTC) and in ambient air (AA). CF-OTC=charcoal-filtered OTC, OZ++OTC=O₃-enriched OTC, AA=ambient air.

The AOT40 exposure calculated in the two different ozone treatment is reported in **Figure 2.1**. The AOT40 value calculated from 1 April to 15 July, for plants grown in O₃-enriched conditions (OZ++OTC), was 21'121 ppb.h, while for those grown under charcoal-filtered air conditions (CF-OTC) was 826 ppb.h. AOT40 in ambient air in the same period was 10676 ppb.h and was calculated using the ozone concentrations recorded by a nearby (6 km) monitoring station of the ARPA Lombardia air quality network (Regional Agency for Environmental Protection).

The critical level of ozone exposure for vegetation (3'000 ppb.h) established by the European Directive 2008/50/EC was already reached on 20 May in the OZ++OTCs while in ambient air it was reached 4 days later.

Table 2. 2 - the starting date of the main phenological stages for five durum wheat cultivars and the start and end of ozone fumigation

	Colombo	Gallareta	Pharaon	Sculptur	Vitrón
Sowing date	9 March	9 March	9 March	9 March	9 March
Plant emergence	21 March	21 March	21 March	21 March	21 March
Plant raising	26 April	23 April	26 April	23 April	23 April
Start O₃ fumigation	6 May	6 May	6 May	6 May	6 May
Flag leaf emergence	15 May	12 May	15 May	12 May	12 May
Earing	25 May	20 May	22 May	20 May	20 May
Anthesis	2 June	29 May	30 May	30 May	29 May
End O₃ fumigation	30 June	30 June	30 June	30 June	30 June
Harvest	15 July	15 July	15 July	15 July	15 July

Table 2.2 reports the starting date of the main phenological stages for each cultivar during the growing season.

2.3.2 Leaf visible injuries and histochemical observations

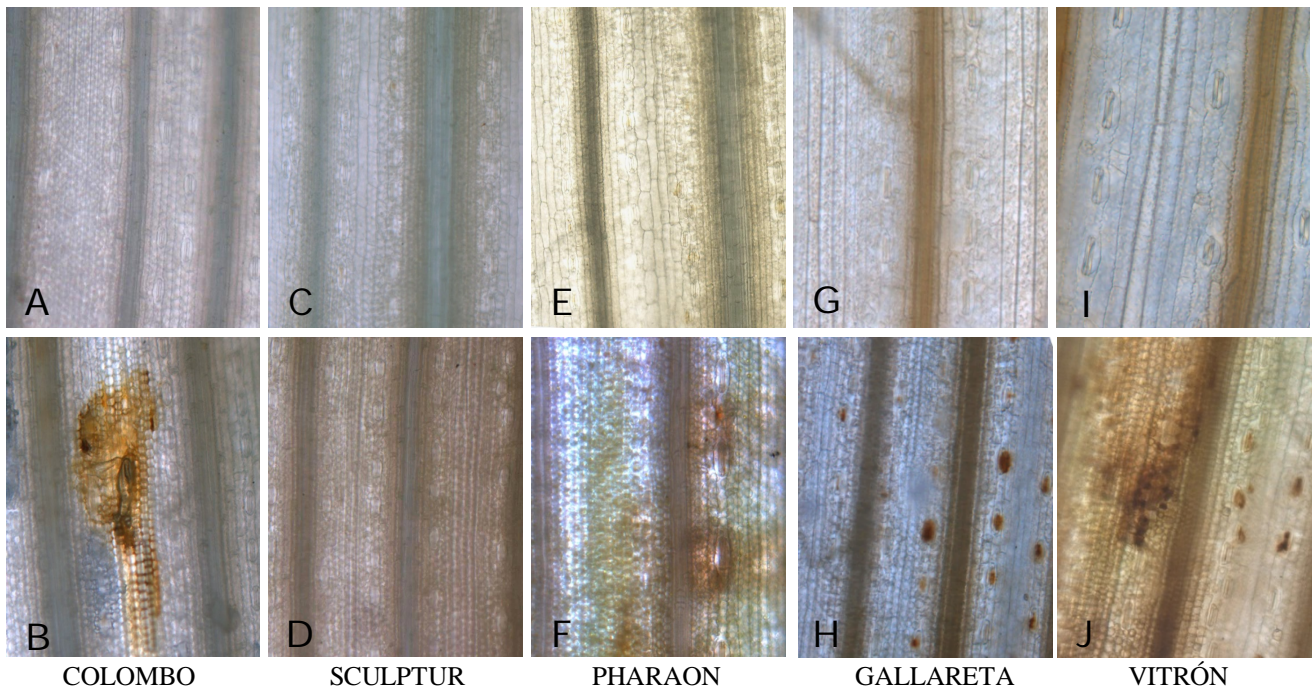


Figure 2. 2- Durum wheat leaves stained with DAB (3,3' diaminobenzidine), cv. Colombo grown in CF-OTC (A) and in OZ++OTC (B), cv. Sculptur grown in CF-OTC (C) and in OZ++OTC (D), cv. Pharaon grown in CF-OTC (E) and in OZ++OTC (F), cv. Gallareta grown in CF-OTC (G) and in OZ++OTC (H), cv. Vitrón grown in CF-OTC (I) and in OZ++OTC (J).

The onset of symptoms in OZ++OTCs was detected in all wheat cultivars, except for Sculptur, the second week of May (**Table 2.3**). They consisted of small chlorotic spots (Picchi *et al.*, 2010), more evident in Gallareta and Vitrón and quite faint in Colombo and Pharaon. However, while in Gallareta, Pharaon and Vitrón they did not get worse in the following weeks, in Colombo they became more severe and they appeared as necrotic lesions at the beginning of June (**Table 2.3**). Sculptur did not show any symptoms up to the end of May, then chlorotic spots appeared suddenly. No significant symptoms were observed in all plants grown in CF-OTCs up to the beginning of leaf senescence in the middle of June. The lack of symptoms in leaves from plants grown in CF-OTCs matched with the absence of hydrogen peroxide (H_2O_2) accumulation in the tissues, as evidenced by Diaminobenzidine (DAB) and light microscope observations (**Figure 2.2, A C E G H**). On the contrary, the plants exposed to high levels of ozone in OZ++OTCs revealed already on 15 May significant H_2O_2 deposits in or around the guard cells and in the mesophyll cell walls nearby. These deposits were restricted to stomata and nearby cells in Gallareta, while

they were diffused also to mesophyll cells in Vitrón, Pharaon, and Colombo (**Figure 2. 2, B, F, H, J**). In symptomless Sculptur's flag leaves they were almost absent at this observation date (**Figure 2. 2, D**). H_2O_2 deposits, indicative of oxidative stress, are at the onset of cell death caused by ozone and responsible for the appearance of visible leaf injury (Faoro and Iriti, 2009).

Table 2. 3 - Ozone-like visible symptoms in the 5 durum wheat cultivars grown in O_3 -enriched OTCs. DAO= days after the start of O_3 -fumigation; - = no symptoms; \pm = small faint chlorotic spots; + = chlorotic spots; ++ = necrotic spots; no symptoms were detected in any of the cultivars growing in CF-OTCs.

Date DAO	15 May 10	28 May 23	06 June 32
Colombo	\pm	+	++
Gallareta	+	+	+
Pharaon	\pm	\pm	\pm
Sculptur	-	+	+
Vitrón	+	+	+

2.3.3 Crop yield

Table 2.4 reports the values of crop yield parameters for each durum wheat cultivar in the two different ozone treatments (CF-OTC and OZ++OTC), while the results of the ANOVA test for the general split-plot design applied are showed in **Table 2.5**, together with the planned comparison on the ANOVA results to show the significance of the response of each cultivar to the different ozone treatments. The ANOVA test shows that the cultivar factor had a highly statistically significant ($p < 0.01$ or $p < 0.001$) influence on the response of durum wheat to all of the listed parameters, except for the total aboveground biomass; ozone alone had a significant influence ($p \leq 0.05$) on the grain yield, aboveground biomass, harvest index and on the dry weight of stems. This latter parameter is also significantly influenced by the interaction between the two factors (see " O_3 * cultivar" column, **Table 2.5**), together with the number of empty (i.e. unfertile) spikes.

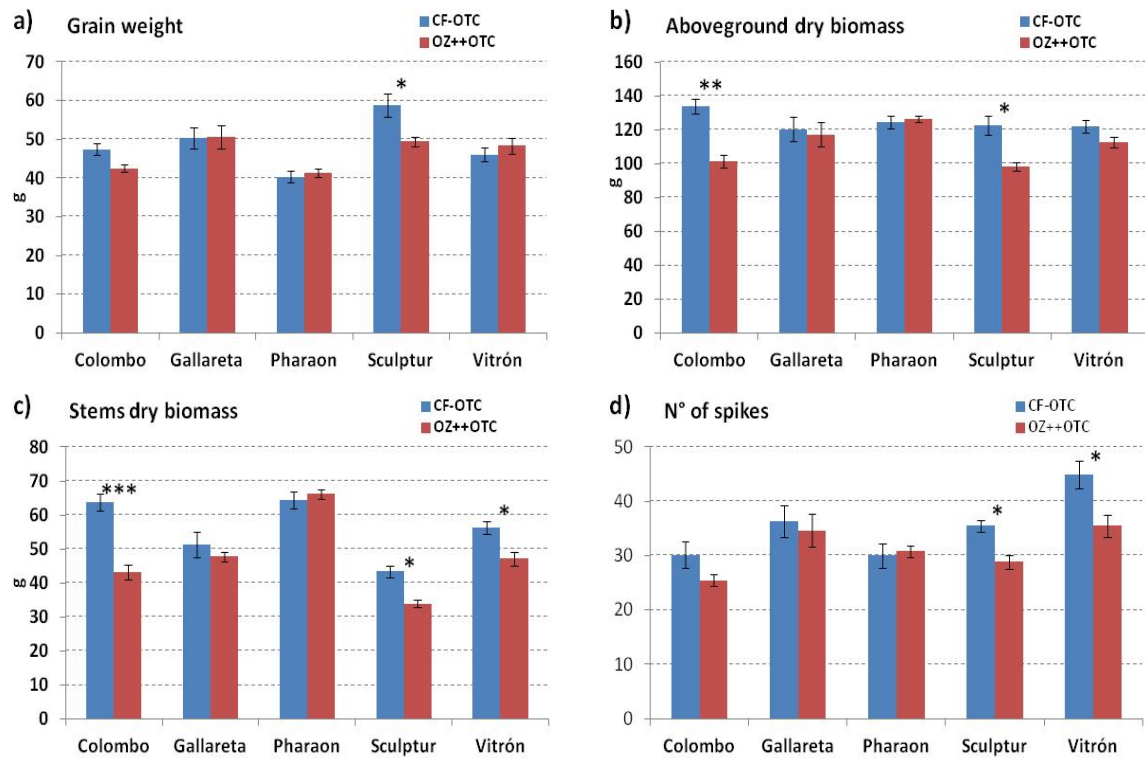


Figure 2.3- Mean weight of grain per pot (a), aboveground biomass per pot (b), mean dry weight of stems per pot (c) and mean number of spikes per pot (d) of the five cultivars of durum wheat grown in charcoal-filtered OTC (CF) and O₃-enriched OTC (OZ++). The bars indicate the standard deviation. Stars indicate statistical significance of the difference between CF-OTCs and OZ++OTCs (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

Analyzing the results of the planned comparison test (last five columns of **Table 2.5**) and the mean values of the main crop yield parameters for each cultivar (**Figure 2.3**), it is worth noting that only the cultivar Sculptur showed a significant decrease of the mean grain weight (-16%, $p = 0.0403$). This cultivar was significantly influenced by the O₃-enriched treatment also in the aboveground biomass (-19.7%, $p = 0.0178$), in the dry weight of stems (-22%, $p = 0.0392$) and in the number of spikes and empty spikes produced (-19%, $p = 0.0481$ and -81% $p = 0.0199$ respectively). Colombo cultivar showed a significant decrease of the aboveground biomass due to ozone (-25%, $p = 0.0039$), that was undoubtedly driven by the decrease of the dry weight of stems (-32%, $p = 0.0007$) and, to a lesser extent, by the decrease of the grain weight (-10%, ns).

Cultivar Vitrón was significantly affected by ozone in the dry weight of stems (-16%, $p = 0.0442$) and in the number of spikes and empty spikes produced, with an important decrease in both parameters (-21% $p = 0.0105$ and -81% $p = 0.0001$).

Finally, Gallareta and Pharaon seemed to be decidedly tolerant to ozone as they didn't show any significant negative effect due to O₃ treatment on any of the parameter considered under the conditions of the present experiment.

Table 2. 4 - Mean values per pot and standard deviation (SD) of yield and growth parameters for the 5 different durum wheat cultivars. (CF= Charcoal-Filtered OTC; OZ++= O₃-enriched OTC).

	O ₃ treat	Grain weight (g)		Aboveground Biomass (g)		Harvest index (%)		Dry weight of stems (g)		N° of spikes		N° of empty spikes		Plant Height (cm)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Colombo	CF	47.5	±4.0	134.1	±10.8	35.4	±1	63.8	±6.0	30.2	±5.0	1.5	±1.0	72.5	±2.7
	OZ++	42.5	±4.7	101.5	±9.2	41.9	±3	43.2	±5.3	25.5	±2.4	1.2	±1.5	67.5	±2.7
Gallareta	CF	50.2	±6.7	120.5	±18.2	41.7	±1	51.3	±9.2	36.3	±7.4	3.8	±2.2	72.5	±2.7
	OZ++	50.6	±11.1	117.3	±17.7	43.1	±4	47.8	±3.6	34.7	±7.3	2.2	±2.2	70.0	±0.0
Pharaon	CF	40.2	±3.7	124.6	±9.7	32.3	±2	64.5	±6.0	30.0	±5.7	1.7	±0.8	67.5	±2.7
	OZ++	41.2	±2.7	126.6	±4.9	32.5	±2	66.2	±3.4	30.8	±2.7	1.5	±2.0	70.0	±0.0
Sculptur	CF	58.8	±7.6	122.7	±13.5	48	±2	43.4	±4.4	35.5	±2.2	2.7	±0.5	60.0	±0.0
	OZ++	49.4	±2.7	98.5	±5.8	50.1	±1	33.9	±2.8	28.8	±3.2	0.5	±0.8	57.5	±2.7
Vitrón	CF	46.1	±4.4	122.2	±8.9	37.7	±2	56.2	±4.5	45.0	±5.0	6.7	±2.3	70.0	±5.5
	OZ++	48.4	±4.8	112.9	±7.5	42.9	±3	47.1	±5.1	35.5	±5.0	1.3	±1.0	70.0	±5.5

Table 2. 5 - Statistical significance of yield parameters for the 5 durum wheat cultivars (split-plot ANOVA with ozone as main factor and cultivar as sub-plot factor). The five last columns indicate the statistical significance of the differences due to ozone in the agronomic parameters for each cultivar (individually tested). Difference between treatments is significant for * p≤0.05, ** p≤0.01 and * p≤0.001. ns=non significant difference.**

	Ozone		Cultivar		O ₃ *Cultivar		Colombo		Gallareta		Pharaon		Sculptur		Vitrón	
	P		P		P		P		P		P		P		P	
Grain Weight	0.0161	*	0.0099	**	0.2568	ns	0.2340	ns	0.9304	ns	0.8021	ns	0.0403	*	0.5611	ns
Aboveground Biomass	0.0371	*	0.2371	ns	0.0767	ns	0.0039	**	0.7007	ns	0.8100	ns	0.0178	*	0.2865	ns
Harvest index	0.0354	*	0.0000	***	0.0510	ns	0.0017	**	0.5322	ns	0.8634	ns	0.1388	ns	0.0057	**
Dry weight of stems	0.0498	*	0.0001	***	0.0301	*	0.0007	***	0.3905	ns	0.6725	ns	0.0392	*	0.0442	*
N° of spikes	0.0683	ns	0.0022	**	0.1846	ns	0.1414	ns	0.5761	ns	0.7782	ns	0.0481	*	0.0105	*
N° of empty spikes	0.1087	ns	0.0039	**	0.0073	**	0.6674	ns	0.0563	ns	0.8291	ns	0.0199	*	0.0001	***
Plant Height	0.2048	ns	0.0011	**	0.4258	ns	0.1053	ns	0.3880	ns	0.3880	ns	0.3880	ns	1.0000	ns

2.3.4 Stomatal conductance and chlorophyll *a* fluorescence

Mean values of stomatal conductance to water vapor detected during each cycle of measurement are reported in **Table 2.6** according to the different ozone treatments experienced by the plants. These values are referred to durum wheat in general, regardless of the different cultivars and of the different days of measurements. The O₃-enriched treatment (OZ++OTC) compared to the CF-OTC treatment, caused a general reduction of g_s in all of the measurement cycles, with a more intense effect at midday and afternoon (-22% and -33% respectively), the ANOVA test performed on these data shows also that these reductions were statistically significant ($p=0.00026$ at midday and $p=0.0000$ at afternoon).

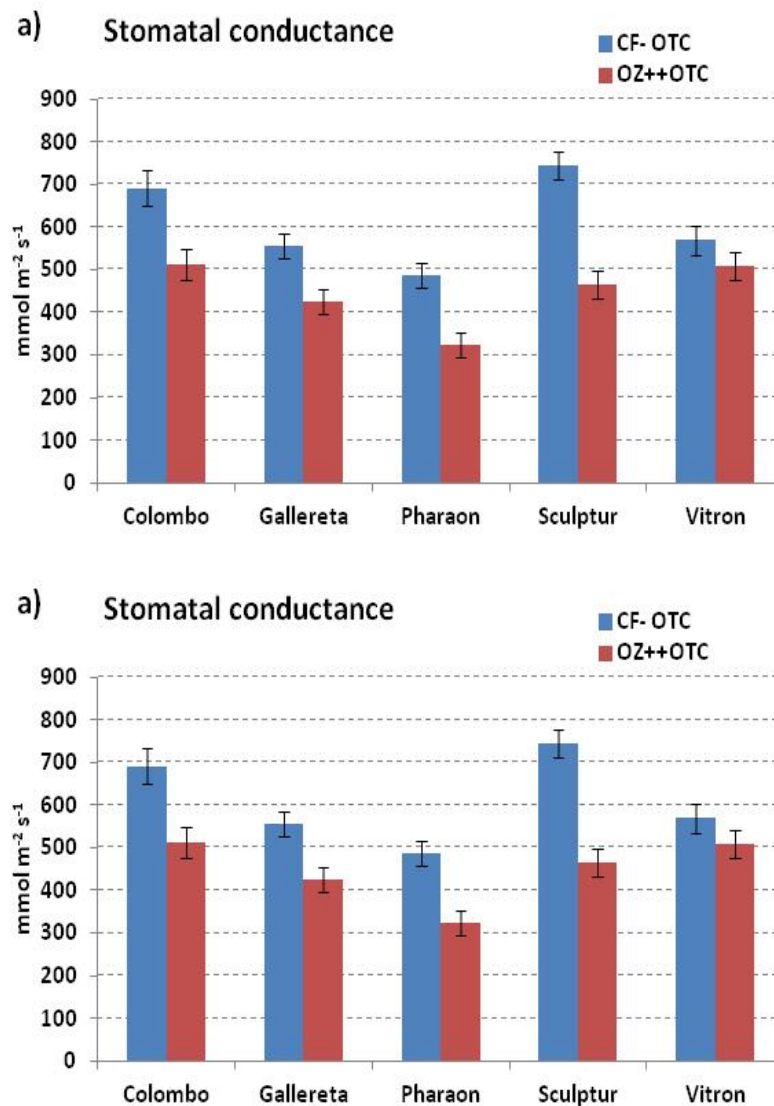


Figure 2. 4 - Mean values of the stomatal conductance to water (a) and Performance Index of photosystem II (b) measured in the different cultivars and ozone treatments, during the growing season. CF=charcoal-filtered OTC, OZ++=O₃-enriched OTC. Bars indicate the standard deviation.

The cultivar-specific response of the g_s to ozone, regardless of the measurement cycles, is reported in **Figure 2. 4(a)**. Sculptur and Colombo, the most affected cultivars by O_3 exposure in terms of growth and productivity, were characterized by the highest mean values of g_s in CF-OTC conditions (748 and 698 $\text{mmol m}^{-2} \text{s}^{-1}$ respectively), but also by the most intense decline in OZ++OTC conditions (-36.3% and -23.9% respectively). Statistical analysis of the differences between OZ++ and CF-OTCs showed that g_s in Colombo, Sculptur and Pharaon, was significantly reduced by the presence of ozone ($p < 0.01$, data not shown), and that this effect was mainly driven by a decline in the afternoon. **Table 2.7** reports in fact, the mean g_s for each cultivar in different cycles of measurement and the statistical significance of the difference due to ozone treatment (one-way ANOVA).

In all of the cultivars except for Vitrón the afternoon decline of g_s in the O_3 -enriched plants was found to be statistically significant, with Sculptur presenting significant decrease also in the morning and midday cycles.

Regarding the effect of ozone on the Performance Index of photosystems, it is evident from **Figure 2.4 (b)** and from the statistical analysis of the measurements (data not shown) that there is no significant damage on the light dependent processes of photosynthesis and that the genetic-related variability found in the values of PI_{abs} seems to be more important than the difference due to ozone exposure.

Table 2. 6 - Mean values of stomatal conductance, standard deviation and statistical significance at different times (cycles) for the 5 durum wheat cultivars. CF-OTC=Charcoal-Filtered OTC; OZ++OTC= O_3 -enriched OTC, SD=Standard Deviation. Difference between treatments is significant for * $p \leq 0.05$, ** $p \leq 0.01$ and * $p \leq 0.001$. ns=non significant difference.**

Cycles	CF-OTC		OZ++OTC		O ₃ effect	<i>p</i>	
	<i>(mmol.m⁻² s⁻¹)</i>	±SD	<i>(mmol.m⁻² s⁻¹)</i>	±SD			
Morning	537	±198	442	±200	-18%	0.0959	ns
Midday	742	±257	581	±263	-22%	0.0003	***
Afternoon	538	±266	358	±222	-33%	0.0000	***

Table 2. 7 - One-way ANOVA test for the difference in g_s values measured at different times of the day between the two ozone treatments. Each cultivar was tested individually. Difference between treatments is significant for * $p \leq 0.05$, ** $p \leq 0.01$ and * $p \leq 0.001$. ns=non significant difference.**

	Cycle	CF-OTC		OZ++OTC		O ₃ effect	p
		(mmol m ⁻² s ⁻¹)	±SD	(mmol m ⁻² s ⁻¹)	±SD		
Colombo	<i>morning</i>	689	±238	561	±239	-18.6%	0.13008
	<i>midday</i>	835	±310	672	±294	-19.5%	0.14624
	<i>afternoon</i>	569	±319	359	±189	-36.9%	0.01338 *
Gallareta	<i>morning</i>	522	±173	493	±219	-5.6%	0.71275
	<i>midday</i>	633	±110	488	±147	-22.9%	0.02339 *
	<i>afternoon</i>	515	±208	325	±179	-36.9%	0.00822 **
Pharaon	<i>morning</i>	457	±154	391	±203	-14.4%	0.30316
	<i>midday</i>	569	±113	421	±246	-26.0%	0.05167
	<i>afternoon</i>	446	±259	199	±159	-55.4%	0.00042 ***
Sculptur	<i>morning</i>	671	±191	482	±192	-28.2%	0.01187 *
	<i>midday</i>	889	±95	576	±211	-35.2%	0.00029 ***
	<i>afternoon</i>	685	±251	371	±254	-45.8%	0.00026 ***
Vitrón	<i>morning</i>	448	±131	399	±172	-10.9%	0.42526
	<i>midday</i>	759	±149	730	±175	-3.8%	0.73478
	<i>afternoon</i>	510	±210	425	±197	-16.7%	0.21510

2.4 Discussion

The present study investigated the response to below and above ambient ozone concentrations of five cultivars of irrigated durum wheat grown in OTC in Mediterranean conditions.

Ozone effects reported in this study was obtained from an OTC experiment subject to methodological advantages and disadvantages. OTC modifies the microenvironment where plants grow, which may interact with its responses to O₃ (Heagle *et al.*, 1988). Despite these uncertainties, the OTC technique was widely used to measure crop responses to air pollutants under realistic O₃ concentrations (Heck *et al.*, 1988; Pleijel *et al.*, 2007; Mills *et al.*, 2007; Feng and Kobayashi, 2009) and comparisons of OTC experimental results and free air O₃ enrichment systems support the conclusions obtained with OTC (Morgan *et al.*, 2006). As an advantage, OTC can be used to significantly reduce ambient O₃ levels and measure plant behavior under pristine conditions even in polluted areas. In this experiment, OZ++OTC O₃ treatment can be considered representative of O₃ polluted rural areas in Mediterranean countries (Paoletti and Manning, 2007; EEA, 2013) while the CF-OTC O₃ concentrations over the experimental period fall well under the critical level established

within the CLRTAP and the EU for the protection of agricultural crops (CLRTAP, 2010; EU, 2008).

Most of the wheat cultivated in the Mediterranean region grows in water limited rain fed areas (Acevedo *et al.*, 1999). The irrigation applied in this experiment has the potential to modify crop responses to O₃ compared with rain fed conditions via increases in g_s and O₃ absorbed through stomata, which are the most damaging for plant physiology since cuticular deposition at close to ambient O₃ concentrations is considered innocuous for plants (Fiscus *et al.*, 2005). Drought stress can ameliorate O₃ damages on wheat (Feng *et al.*, 2008). Thus, the results from the present experiment must be regarded as a worst case scenario, where no limiting environmental conditions for O₃ uptake occur as opposed to limiting conditions in the field. However, the nature of the interactive effects of drought and O₃ on crops yield and growth is complex. Under some instances drought may not reduce O₃ effects and O₃ can increase the vulnerability of plants to water scarcity (Wilkinson *et al.*, 2012; Alonso *et al.*, 2013). Furthermore, trade-offs in yield may exist if irrigation is reduced under water limiting conditions for crop production in order to reduce O₃ fluxes and associated effects.

The physiological and yield response of durum wheat to O₃ exposure was cultivar dependent. Sensitive and resistant cultivars could be identified. Two sensitive cultivars, Sculptur and Colombo, showed a reduction of aboveground biomass growth and yield, though only Sculptur showed a statistically significant reduction in grain yield (-16%, $p=0.04$). Gallareta and Pharaon were not affected by O₃ in biomass or yield parameters, even showing a slight increase of grain weight per pot of +1 and +2% (n.s.) respectively. Vitrón behaved like a moderately tolerant cultivar, with reductions in dry weight of stems and number of spikes, compensated by a strong reduction of the number of empty spikes and a stimulated grain yield, resulting in a no statistically significant stimulation in yield of +5%.

Durum wheat yield losses due to a direct effect of O₃ have been previously confirmed in O₃ fumigation experiments carried out in OTC. One experiment with a durum wheat cultivar in Northern Italy showed a similar response than other experiments with common wheat cultivars in Northern and Central Europe (Pleijel *et al.*, 2007) while other O₃ fumigation experiments have shown that some durum wheat cultivars are O₃-tolerant (Badiani *et al.*, 1996; Gerosa *et al.*, 2012, 2014; Reichenauer *et al.*, 1998). Moreover, De Marco *et al.* (2010) did not find a statistically significant relationship between durum

wheat yield and calculated POD (Phytotoxical Ozone Dose) across Italy once the effect of other environmental factors on wheat yield were considered.

Interestingly, irrespective of the sensitivity to ozone of the growth and yield parameters, all the cultivars consistently increased their harvest index under high O₃ as compared with low O₃ OTC.

A general slight increase in harvest index was found in all the plants exposed to ozone (OZ++OTCs) and was most important and significant in Colombo (+18%, $p=0.0017$) and Vitrón (+13%, $p=0.0057$) two cultivars showing also a strong reduction in some growth parameters (dry weight of stems) due to O₃ but no discernible effects on yield (**Table 2.4**).

A stimulation of aboveground growth and/or reproductive development due to ozone has been previously described in other herbaceous species as a compensation mechanism in response to abiotic stress (Black *et al.*, 2000; Davison and Barnes, 1998; Fiscus *et al.*, 2005; Gerosa *et al.*, 2009). These compensation mechanisms could explain the apparent differences in sensitivity between growth and yield parameters observed in cultivars Colombo and Vitrón.

Inter and intra-specific variability in O₃ sensitivity has been well described for wheat and other crop species (Fiscus *et al.*, 2005; Mills *et al.*, 2007; Biswas *et al.*, 2008b; Betzelberg *et al.*, 2010; González-Fernández *et al.*, 2010, 2014; Wilkinson *et al.*, 2012). Ozone sensitivity is considered a heritable trait and its variability was often related with differences in stomatal conductance, photosynthetic capacity, detoxification capacity or volatile organic compound emissions (Fiscus *et al.*, 2005; Wilkinson *et al.*, 2012; Loreto and Fares, 2007).

In this experiment, sensitive cultivars Sculptur and Colombo and moderately tolerant Vitrón showed the highest g_s levels in both O₃ treatments compared to tolerant cultivars Gallareta and Pharaon (**Figure 2. 3a**). This would be consistent with higher O₃ stomatal fluxes related to stronger O₃ effects. Several studies identified similar associations between g_s rates and cultivar variability in O₃ sensitivity (Pleijel *et al.*, 2006; Biswas *et al.*, 2008b) or reduction of O₃ effects due to lower g_s induced by elevated CO₂ concentration or drought (Fiscus *et al.*, 2005; Feng *et al.*, 2008; Fagnano *et al.*, 2009). However, others failed to find a clear relationship between g_s and variability in O₃ sensitivity (González-Fernández *et al.*, 2010; Feng *et al.*, 2011), pointing that other factors also determine intra-specific changes in O₃ sensitivity (Feng *et al.*, 2010; Fiscus *et al.*, 2005; Wilkinson *et al.*, 2012).

Ozone exposure caused a general reduction of g_s in all of the cultivars, and this decrease was more pronounced and significant at midday and in the afternoon (**Table 2.7**), probably as a consequence of the daily ozone detrimental action that might have caused stomata impairment at this time of the day, after plants had already experienced several hours of active ozone fumigation. Moreover, O_3 induced g_s reductions were the highest in sensitive cultivars Sculptur and Colombo. A similar result was found by Feng *et al.* (2011) in common wheat, where O_3 reduced g_s of the sensitive cultivar, but not of the tolerant one.

Thus stomatal conductance, as confirmed by other studies (Danielsson *et al.*, 2003; Sarkar and Agrawal., 2012; Pleijel *et al.*, 2006), seemed to play a key role in the response of plants to ozone in terms of productivity.

Ozone induced reductions in g_s are generally considered to be the result of reduced photosynthesis/assimilation or direct damages on guard cells (Fiscus *et al.*, 2005). However, it is important to highlight that the statistical analysis showed no significant effects of O_3 on the Performance Index of the photosystem II in all of the cultivars. This result was somehow surprising because we expected a clear effect of ozone on the photosynthesis, at least on the most sensitive cultivars in terms of grain yield and biomass production. These negative effects were also reported in many studies on other species such as bean and spring wheat (Meyer *et al.*, 2000; Guidi *et al.*, 2000), however other authors did not find clear effects (Herbinger *et al.*, 2002).

Even if the physiological behavior of Sculptur and Colombo can be considered similar in terms of g_s and chlorophyll fluorescence, the histochemical analysis conducted on the flag leaves, pointed out different patterns and mechanisms of damage caused by ozone.

The reduction of g_s in Colombo was undoubtedly related to the presence of widespread damages on the guard cells of stomata (high amount of H_2O_2 deposits, **Figure 2.2 B**), and, as a consequence, stomatal functionality might have been compromised. Sculptur plants, on the contrary, didn't show any specific H_2O_2 deposits both on the guard and mesophyll cells (**Figure 2.2 C**) suggesting that the g_s reduction detected on OZ++OTC plants is related to a negative effect of ozone on the content and activity of RuBisCO, as found previously in wheat and in other species (Cao *et al.*, 2009; Farage and Long., 1999; Goumenaki., 2010), thus leading to a decreased activity of the Calvin cycle and the consequent increase of the intercellular CO_2 with a negative feedback mechanism on g_s .

Ozone-like visible symptoms support this hypothesis, as they appeared in Sculptur some weeks later than in all the other cultivars, suggesting that slight cell damages occurred,

enough to reduced yield but insufficient to rise adequate H_2O_2 level for eliciting cell death and the subsequent visible symptoms. The latter appeared at the end of May and did not get worse later on as in Colombo. This behaviour also suggests that ROS scavengers in Sculptur could be more efficient than in Colombo and the other examined cultivars, at least at the beginning of ozone treatment. In any case, it must be stressed that sensitivity in terms of visible ozone-like symptoms do not correlate with the final crop yield. In fact, Pharaon and Gallareta which were already symptomatic in the middle of May did not show any significant negative effect the considered yield parameters. The reasons for the lack of correlation, besides what suggested before for Sculptur, could relay in a better capacity of some cultivars to restrain the initial injury, causing localized H_2O_2 accumulation and subsequent chlorotic spots, by rising afterwards stronger antioxidant defences and limiting the damages in most of the mesophyll tissues. Finally, in the plants grown in CF-OTC the onset of symptoms occurred only the second week of June, starting with Colombo e Vitrón, but at this time chlorotic spots were easily confused with incipient leaf senescence that occurred in one more week. Though chlorotic/necrotic spots observed in OZ++OTCs were clearly caused by ozone and not by other types of stressor, in agreement with previous reports (Mishra *et al.*, 2013; Picchi *et al.*, 2010; Nussbaum *et al.*, 2000), their appearance time and severity cannot be used as indicator of ozone sensitivity/tolerance and to predict the crop yield losses.

The physiological measurements performed in this study were not conclusive for explaining intra-specific variability in O_3 sensitivity of durum wheat. Several studies suggest that intra-specific differences in detoxification capacity or interactions between O_3 and other environmental factors determine intra-specific changes in O_3 sensitivity (Feng *et al.*, 2010, 2011; Fiscus *et al.*, 2005; Wilkinson *et al.*, 2012). Moreover, repair and detoxification mechanisms occur at a metabolic cost for the plant (Fiscus *et al.*, 2005), which may be reflected in reduced growth or yield in crops. These factors are probably mediating the differences observed in the response to O_3 of durum wheat cultivars in this experiment.

The results from this study are relevant for evaluating O_3 effects on agricultural production in Mediterranean European countries. The O_3 exposure or dose-response functions currently in use within the CLRTAP to set O_3 critical levels (Pleijel *et al.*, 2007; CLRTAP, 2010; Mills *et al.*, 2011a) and evaluate O_3 effects on agricultural production (Mills *et al.*, 2011b) is skewed towards North and Central European experiments studying common

wheat cultivars. In Southern European countries, wheat is also an economically important crop and durum wheat is as important as common wheat in terms of cultivated surface (EUROSTAT, 2014). In comparison with current exposure-response functions, the O₃ sensitivity of durum wheat cultivars tested in this experiment is lower than common wheat. Expected yield losses under elevated O₃ OTC treatment in this study amounted to 35% compared with a zero AOT40 exposure according with the Mapping Manual exposure-response function (CLRTAP, 2010). Yield losses of the most sensitive cultivar of durum wheat in this experiment scored just 16% under above ambient O₃ exposure compared to CF-OTC. It could be argued that Mediterranean wheat cultivars might be more O₃ tolerant compared with cultivars from more Northern areas of Europe since O₃ tolerance is a heritable trait and natural and artificial selection in breeding sites with high O₃ levels combined with other environmental stresses, like drought or temperature, could result in O₃-tolerant crop cultivars (Barnes *et al.*, 1999; Biswas *et al.*, 2008b). However, some studies found no relationships between O₃ tolerance and ambient O₃ concentrations at the breeding sites nor associations between O₃ and drought tolerance in Chinese common wheat cultivars (Biswas *et al.*, 2009; Biswas and Jiang 2011). The intra-specific variability in O₃ sensitivity of common and durum wheat identified in this and other studies must be considered in risk assessments and quantification of O₃ damages in agricultural production since adaptations at the farm level by selecting tolerant cultivars can help to modulate O₃ impacts. Interestingly, this study shows that the most sensitive cultivar, Sculptur, is also the most productive under below ambient O₃ concentrations. The high productivity of Sculptur under a range of Mediterranean climatic conditions was also confirmed by productivity trials of durum wheat cultivars across Spain (GENVCE, 2010). Less productive cultivars were O₃ tolerant and their grain yield under elevated O₃ in this study was greater than that of Sculptur. At the farm level, a change of Sculptur by O₃ tolerant but less productive cv. Gallareta would result in a decrease of 14% of productivity under elevated O₃, which is slightly less than yield losses expected if Sculptur was maintained (-16%). Under field conditions in Spain, yield of the tolerant cultivar Gallareta was 16% lower than Sculptur (GENVCE, 2010), similar to the 15% difference found in below ambient O₃ OTC of this study. Unfortunately no information of O₃ levels at the trial sites is available to date in order to evaluate the potential severity of O₃ effects in the field trials and compare them with the experimental results.

These results drive to the conclusion that estimated negative effects of O₃ on agricultural production might be less than what previously estimated using current exposure-response relationships based on the response of sensitive common wheat cultivars. Ozone effects on agricultural yield are probably tempered by existing tolerant cultivars/wheat species, environmental conditions as well as agronomic practices, though some may result in indirect yield reductions under current conditions.

Further information is required to define the range of O₃ sensitivity of Mediterranean bread and durum wheat cultivars and perform new field validations before quantifications of O₃ induced wheat yield losses can be performed in the Mediterranean area.

2.5 Conclusions

Durum wheat response to ozone was highly variable depending on the cultivar and parameter considered. The greatest yield reductions were observed in the most productive cultivars when plants were grown in ozone enriched conditions. Therefore, the intraspecific variability in O₃ sensitivity must be accounted for to evaluate O₃ impacts in crop production.

Mitigation of the O₃ effects can be reached by growing O₃ tolerant cultivars, but tradeoffs in yield may also occur in relation to soil water availability conditions.

According to the experimental results of this study, the current O₃ critical levels for vegetation in terms of AOT40 should provide enough protection for durum wheat cultivation in the Mediterranean region, but on the other hand, model-based risk assessments for crop losses in this area could lead to significant overestimations of the ozone damages on durum wheat.

Chapter 3.

Response of two Italian cultivars of durum wheat to ozone exposure and efficacy of chitosan as plant protectant from ozone damages

Manuscript in preparation as:

Monga, R., Marzuoli, R., Gerosa, G., Faoro, F., Finco, A. Response of two Italian cultivars of durum wheat to ozone exposure and efficacy of chitosan as plant protectant from ozone damages.

Abstract

Two cultivars of durum wheat, Colombo and Sculptur, were grown in the Open-Top Chambers. Plants were exposed to four different ozone concentrations: ambient concentration (NF), minus 50% (CF), plus 30% (OZ+) and 60 % (OZ++) in respect to ambient ozone concentration. Two types of chitosan were applied as leaf spraying (chitosan 40 kDa: CHI40, chitosan 300 kDa: CHI300) plus tap water as control (CTL) within each OTC. Non significant effect of ozone exposure was observed in grain yield, chlorophyll fluorescence, Amax, gm* and triphosphate use (TPU) for Sculptur cv, while Colombo cv showed a significant reduction in grain yield, chlorophyll fluorescence, maximum assimilation (Amax) and triphosphate use (TPU) under ozone conditions. The stomatal conductance was significantly reduced by ozone exposure in both durum wheat cultivars. A reduction of maximum rate of Rubisco carboxylation (Vcmax) was also observed in both cultivars, however not significant. Both CHI40 and CHI300 alone induced a significant decrease of stomatal conductance and a non significant decrease of chlorophyll fluorescence and net photosynthesis in both cultivars. No significant differences were found in the effects of the two chitosan types. The combined effect of ozone and chitosan treatment resulted in a reduced grain yield that was significant only in Sculptur. The physiological parameters such as stomatal conductance, chlorophyll fluorescence and net photosynthesis were decreased under combined treatment but nether significantly.

Key words: ozone effect; *Triticum durum* ; tropospheric ozone; chitosan; symptoms; stomatal conductance; chlorophyll fluorescence; grain yield.

3.1 Introduction

Current concentration of tropospheric ozone affects constantly the agricultural crop production by impairing the stomatal functioning, altering the leaf gas exchanges, slowing plant growth, accelerating leaf senescence and decreasing consequently the economic yield (Ojanperä *et al.*, 1992; Vlachokostas *et al.*, 2010; Fumagalli *et al.*, 2001; Van Dingenen *et al.*, 2009). Additionally, the elevated ozone concentration causes deleterious effects on crops in decreasing photosynthetic pigment contents with the duration of ozone exposure, affecting the gas exchange and carboxylation, inducing reductions in light-saturated photosynthesis (Asat), the maximum activity of Rubisco ($V_{c,max}$) and the maximum electron transport rate (J_{max}) and altering assimilate partitioning (Biswas *et al.*, 2008b; Meyer *et al.*, 2000; Chen *et al.*, 2008; Farage and Long, 1999).

The sensitivity of agricultural crops to ozone becomes a concern for more than one researcher fearing food scarcity in a world where the population is expanding rapidly. (Pleijel *et al.*, 2006; Feng *et al.*, 2008; Akhtar *et al.*, 2010a; Wang *et al.*, 2007). Durum wheat, one of the most important crops world-wide (FAOSTAT, 2009; Paulsen and Shroyer, 2004) and adapted to Mediterranean environment conditions (Büker *et al.*, 2007; Corbellini *et al.*, 1997) is not spared from the damages caused by ozone exposure. Study conducted by Picchi *et al.* (2010) on some cultivars of durum wheat demonstrated the effects of ozone exposure on appearance of symptoms and important loss of productivity but these differed from variety to variety.

Plant response to elevated ozone concentrations may be influenced by the atmospheric conditions, genotype factors, cultural practices and could even vary according to year (Emberson *et al.*, 2009; Pleijel *et al.*, 2000; Mills *et al.*, 2000). For example many experiments demonstrated that plant injury cannot occur if environmental and biological conditions are not appropriate for gas exchange and ozone uptake even if ambient ozone concentration is considered high (Piikki *et al.*, 2008; Manning, 2003). Numerous authors reported the ozone-reduced stomatal conductance in wheat exposed to elevated ozone (Balaguer *et al.*, 1995; Cao *et al.*, 2009). Nevertheless other authors demonstrated an enhancement of stomatal conductance or no change of this parameter under ozone conditions (Biswas *et al.*, 2008b; Gerosa *et al.*, 2014). The reduction of grain yield caused by ozone exposure observed in many studies accounts for a severe economic loss (Vlachokostas *et al.*, 2010). Ollerenshaw and Lyons (1999) observed an O_3 -induced yield decrease of 13% for the plant of wheat exposed to an ozone concentration of 80 nmol mol⁻¹

¹. Our previous study carried out with two durum wheat cultivars has suggested a contrasted response between cultivars for the plants exposed to increment of 50% of ambient concentration (Gerosa *et al.*, 2014).

To promote the adaptation of plants to ozone exposure, the genetic selection of more resistant cultivars (cultivars with appropriate biochemical or physiological characteristics) or the plant protection by applying chemicals that could reduce the pollutant dose (antitranspirant application or increment of systemic defenses) would be envisaged (proposed).

Chitosan, one of the antitranspirant compounds is a natural, biodegradable polysaccharide polymer obtained by deacetylation of chitin and currently produced from the exoskeleton of crustaceans such as crabs, krills and shrimps (Sanford, 2002; Rinaudo, 2006; Chen *et al.*, 2014).

Chitosan has multiple uses in agriculture: (i) To minimize the escape of water from the plant by decreasing stomatal conductance, thus reducing transpirational losses, improving plant water status and reducing wilting and leaf abscission (Goreta *et al.*, 2007; Iriti *et al.*, 2009; Gu *et al.*, 1998; Del Amor *et al.*, 2010), (ii) to fight against viral and bacterial diseases (Raafat *et al.*, 2008; Faoro *et al.*, 2001; Iriti *et al.*, 2006), (iii) to possess antifungal properties that enable it to induce morphological changes, structural alterations and molecular disorganization of the fungal cells (EL Ghaouth *et al.*, 1999; Ait Barka *et al.*, 2004; Bautista-Bañosa *et al.*, 2006), (iv) to enhance and regulate the plant growth, development and yield in several crops (O'Herlihy *et al.*, 2003; Cabrera *et al.*, 2013; Wang *et al.*, 2015).

3.2 Objectives

The aim of this experiment was:

- 1) to investigate more deeply the response to O₃ of two durum wheat cultivars (Colombo and Sculptur) that were found particularly O₃ sensitive in the previous varietal screening experiment on 5 durum wheat cultivars (3 Italian and 2 Spanish);
- 2) to check whether there is a correlation between (visible) leaf symptoms, effects at physiological level and productivity in these two Italian cultivars, highly cultivated because very productive, taking into account different exposure levels of ozone;

3) to assess the potential role of the application of an antitranspirant (chitosan, leaf spraying) in protecting wheat plants from ozone damages in order to reduce impacts and negative effects after peak concentration episodes.

3.3 Materials and Methods

3.3.1 Experimental site

The experiments were performed in the Open-Top Chambers (OTC) experimental site of C.R.IN.ES (Research Center on Air Pollution and Ecosystems) which is located inside the Regional Center for the Enhancement of Forest Biodiversity (formerly Forest Nursery) of the Lombardy Region (c/o ERSAF, the Regional Agency for Agriculture and Forestry Services) at Curno, Bergamo (Lat. 45° 41'17'' N, Long. 9°36'40'' E, elev. 242 m a.s.l.) in Northern Italy.

3.3.2 Open-Top Chambers

The Open-Top Chambers (OTCs) where plants were grown in this experiment were 2.4 m in height with a 3 m diameter (Heagle et al. 1973). The scaffold of each Open-Top Chamber was made of an aluminum frame and the outer surface of the frame was covered with two highly transparent plastic (polyvinyl chloride; PVC) covers (one upper and one lower). The upper top of each chamber was open to ensure identical meteorological conditions inside the chambers than outside, or at least very similar. The lower PVC cover consisted of two folded layers, the innermost of which has holes arranged evenly on its surface to allow a uniform distribution – within the chambers - of the air coming from the external ventilation system. Each ventilation system consisted of a metal box that houses an electric single phase motor (model Baldor VL3507-70, 0,75cv) equipped with an aluminum propeller (Mavib), eventually fitted with 12 activated carbon (Comelt) filters to remove ozone from the incoming air. The filtering system enabled an abatement of about 50% of ambient air ozone concentration. The ozone enrichment system consisted of an ozone generator (TS-10, Ozone Solutions Inc., USA) and a set of Teflon pipes to lead the produced ozone directly to the ozone-enriched Open-Top Chambers (OZ++ OTC). Ozone was injected into the air ventilation system and diluted with the ambient air prior to reach the plants.

3.3.3 Plant material

Two cultivars of durum wheat (*Triticum durum*) were sown on March 03, 2014 in 11L pots (25 cm diameter and 23 cm height) filled with 10 kg of standard soil (Koro Excell) composed of peat (80%), composted green (20%), organic C (25%), organic N (0.8%), and with a C/N ratio of 31, salinity (1 dS/m), bulk density of 16kg/80L) and pH (5.5-6.5). Three plants of the same cultivar were grown in each pot. Plants entered into anthesis phase 76 and 78 days after sowing (DAS) for Sculptur and Colombo respectively. Both cultivars were harvested 118 DAS. Plants were fertilized twice during the experiment with urea 46%, 39 days (1 gram per pot) and 70 days (0.5 gram per pot) after sowing date. Plants were automatically irrigated using an overhead sprinkler irrigation system (one sprinkler head in each OTC) to get a constant soil moisture around field capacity (40% in volume).

3.3.4 Experimental treatments:

3.3.4.1 Ozone

Pots were randomly placed in 12 OTCs (18 pots per cultivars for a total of 36 pots in each OTC), 46 days after sowing. The OTCs were arranged in 3 blocks of 4 OTCs corresponding to 4 different levels of ozone treatments. During this experiment, four levels of ozone fumigation were applied: i) charcoal-filtered OTC (CF) in which ambient air was filtered of about 50% of ozone concentration by charcoal filters, ii) non-filtered OTCs (NF) where the ambient air was let in without filtering, iii) moderate ozone supplemented OTCs (OZ+) in which non-filtered air was incremented by 30% in ozone concentration and iv) high ozone supplemented OTCs (OZ++), in which the increment in ozone concentration was 60%. Ozone for the fumigated OTCs was generated using an ozone generator (TS-10, Ozone Solutions Inc., USA) fed with pure oxygen produced by an oxygen generator (OG-20, Oxygen Generating System., USA). The ozone within each chamber was monitored continuously through sequential sampling air controlled by an automatic solenoid valves conveying air to ozone analyzer (model 1108 RS, Dasibi Italia s.r.l., I). This analyzer was calibrated at the beginning and end of the experiment and the data corrected accordingly. Ozone sampling points were set at 100 cm height in the centre of OTCs, just at the canopy top level, as required by Mapping Manual for the calculation of the AOT40. The system was managed by a dedicated personal computer equipped with a NI-DAQ 6.9 I/O board (National Instruments, Austin, TX) and a Labview 6.1 program devised specifically for this experimentation. The data acquisition for all sensors were performed automatically every hours by two data loggers (CR10 x and CR1000 with AM

16/32 multiplexing system, Campbell Scientific Inc., UK), and then downloaded to a common Personal Computer. Ozone treatments were applied in three replications (expressed as blocks).

3.3.4.2 Chitosan

Within all OTCs, pots of each cultivar were subdivided in three groups. Plants from the first group were sprayed with tap water (CTRL), those from the second group with chitosan 40 kDa (CHI40, low molecular weight), and plants from the last group with chitosan 300 kDa (CHI300, high molecular weight). Both chitosans were bought from Sigma-Aldrich, USA, and had a deacetylation degree (DD) of about 85%. Chitosan solutions were prepared at the final concentration of 0,05% (w/v) by dissolving overnight chitosan powder in 0.01% of acetic acid and adjusting pH at 5.6.

3.3.5 Ozone exposure

Exposure of plant to ozone was expressed as AOT40 (accumulated ozone exposure threshold of 40 ppb.h) was calculated as the sum of hourly ozone concentrations above a cut-off threshold of 40 ppb during daylight hours (between 8:00 and 20:00, when global radiation exceeds 50 W m^{-2}) over the experimental time period (Kärenlampi and Skärby, 1996).

$$AOT40 = \sum_{\substack{\forall [O_3] > 40 \text{ ppb} \\ \forall Rad \geq 50 \text{ W/m}^2}} ([O_3] - 40) \cdot \Delta t$$

where $[O_3]$ is the hourly ozone concentration expressed as ppb, and t is the running hours during the time of evaluation. The AOT40 unit is ppb·h. The comparability of AOT40 exposures between different plants is possible only in optimal water supply for the phytosphere (i.e. soil moisture at field capacity) in order to exclude possible limitations of ozone uptake due to stomatal closure (Fuhrer and Achermann, 1994). The Mapping Manual suggest an accumulation period for the AOT40 calculation is set from 1 May to 31 July for all the European crops. However in this study, AOT40 was calculated for the whole experimental period, i.e. between 28th March to 18th June 2014.

3.3.6 Agrometeorological measurements.

Air temperature and humidity (50Y, Campbell Scientific Inc., Logan, Utah), wind speed and direction (WSS1&2, Environmental Measurements LTD, UK), global radiation and photosynthetically active radiation (210SZ and 190SZ, LiCOR, USA) and rain (Young,

USA) were measured under ambient conditions. Additionally the thermo-hygrometers (Campbell), PAR sensors (LiCOR), anemometers (Environmental Measurements), and rain gauges (Young) were also installed in some OTCs, just in the middle of them on a wood pole at 100 cm of height. Soil moisture sensors (EC5 Decagon, USA) have been installed in two pots not treated with chitosan within each OTC for measuring volumetric moisture content of soil. In the same pots soil temperature was monitored (GMR strumenti, Italia). Meteorological parameters were measured continuously as hourly mean for each OTC. The datalogger (CR1000 with AM 16/32 multiplexing system, Campbell Scientific Inc., UK) was used for meteorological data acquisition.

3.3.7 Leaf visible symptoms assessment

Ozone visible symptoms were monitored every day from the starting of ozone fumigation looking for the onset of chlorosis and necrosis symptoms that are typical of O₃ injury on durum wheat (Picchi *et al.*, 2010). Once the first visible symptoms appeared, the diffusion of the symptoms has been assessed by taking regularly pictures with a common PC scanner of 8 randomly chosen leaves from each plot. Four samples of flags leaves (2 leaves for cv Colombo and 2 leaves for cv Scultpur) were randomly collected from each charcoal-filtered (CF-OTC) and highly O₃-enriched (OZ++OTC) Open-Top Chamber, twice during the growing season (May 14th and 27th). In total, 48 leaves were digitalized with a scanner and visually examined for the presence of macroscopic symptoms. The collected flags leaves were also analyzed using Global Lab (Data Translation, Marlboro, MA, USA) to more accurately determine the percent of chlorotic/necrotic area per cm².

3.3.8 Ecophysiological analysis

3.3.8.1 Stomatal conductance measurements

Stomatal conductance to water vapor was measured with a Delta-T diffusion porometer (AP4, Delta-T, UK), that was calibrated before each measurement cycle. Analysis were performed on nine flag leaves of plants for each cultivar randomly chosen: three sprayed with tap water (CTRL), three sprayed with chitosan 40 kDa (CHI40) and three sprayed with chitosan 300 kDa (CHI300). Four measurements campaigns of Stomatal conductance were carried out during the growing season 65 days, 73 days, 83 days, and 97 days after sowing date. Three different daily cycles of measurements were performed (between 7.00-9.00 12.00-14.00 and at 16.00-18.00, for morning cycle, midday cycle and afternoon cycle respectively).

3.3.8.2 Chlorophyll fluorescence measurements

Chlorophyll fluorescence parameters measurements were performed on nine wheat flag leaves (three flag leaves per chitosan treatment) per each cultivar randomly chosen per each OTC. Plants flag leaves were dark adapted with leaf clips 20-30 minutes prior to be measured. Chlorophyll fluorescence parameters were measured three times during the growing season 69, 80 and 89 days after sowing date (once a day, in the morning between 10.00 and 12.00). Measurements were taken using a Handy PEA fluorimeter (Hansatech Instruments Ltd, UK).

3.3.8.3 Gas exchange measurements

The rate of CO₂ assimilation (A) to changing intercellular CO₂ concentration (C_i) was measured with a portable infra-red gas analyzer CIRAS 2 (PPSystems, USA) in plants belonging to the CF and OZ++ OTCs. Six plants in each OTC (three for each cultivar) were chosen from the CTRL chitosan treatment. These six plants were measured two times, 8 and 31 May, respectively. During the measurements, the cuvette environment was maintained with a relative humidity between 60 and 65% and a PAR intensity of 1800 mmol of photons m⁻²s⁻¹, while leaf temperature was set at 25 °C. All measurements were conducted between morning and midday. The A/C_i curves were constructed following the methodology of Sharkey *et al.* (2007) to calculate the maximum rate of Rubisco carboxylation (V_{cmax}), the maximum rate of photosynthetic transport (J), the mitochondrial respiration (R_d), and the triose phosphate use (TPU).

3.3.9 Biomass and Yield determination

The harvest occurred 108 days after plant sowing (18 June). Plant height for each cultivar was taken in different ozone and chitosan treatments before proceeding to harvest. All the plants of a single pot were cut and weighted (total fresh weight). Then, the total number of spikes per plant and the number of empty spikes per plant were determined. Stems were separated from spikes and weighted separately to obtain fresh weight of stems and spikes. Stems were dried in an oven at 80°C for 72 hours and reweighed (dry weight of stems). Spikes were kept in a controlled dry ambient for two months and reweighed (dry weight of spikes). After shelling the spikes, the grain yield was assessed for each single pot. The hectolitre weight of seeds was determined for both cultivars grown in the same conditions (different ozone and chitosan treatments) using the Grain Analyzer Computer (GAC 2000,

Dickey-John corp., USA). Harvest index (HI) was calculated as the ratio between grain weight per pot and total aboveground biomass per pot.

3.3.10 Statistical analysis

Data were analyzed according to a complete split-plot design with randomized blocks, considering 4 levels of the main factor (O_3), 3 levels of the nested factor (chitosan) and 3 replicates (Blocks). Statistical analysis were performed separately for each cultivar using the GLM (General Linear Model) module of STATISTICA 8.0 software (Tulsa, USA). The significance of the difference between different treatments was tested for yield, stomatal conductance, chlorophyll fluorescence and net assimilation (P_n). A one-way ANOVA was used to test the effect of ozone on the maximum assimilation of CO_2 (A_{max}), the maximum rate of Rubisco carboxylation (V_{cmax}), the maximum rate of photosynthetic transport (J), the day respiration (R_d^*), and the triose phosphate use (TPU).

3.4 Results

3.4.1 Meteorological conditions

Table 3.1 reports the main meteorological variables recorded in ambient air and OTC during the experiment and the ozone concentrations (max and average) in ambient air during the same period. The values of average temperature, precipitation and average ozone concentration were quite similar for April and May, while June showed the increment of the mean temperature (+4.6°C and 5°C compared to May in AA and in OTC respectively) and consequently the increase of ozone concentration in AA (+10 ppb as general mean compared to May). The significant increase of precipitation recorded in June compared to the values of other months, is due mainly to an episode of extreme rainfall that occurred between 25th and 26th June (shortly before harvesting) with 80 mm recorded in only 14 hours (more than the half of total monthly value). The levels of precipitation for the rest of the month were similar to those of the previous months. The data in the table show further the presence of the chamber effect of the OTCs (already known), estimated on an average of 24 hours (estimated as day average) of 1 ° C and 7% of temperature (T) and relative humidity (RH) respectively.

Table 3. 1- Monthly rainfall, average temperature, average relative humidity, average photosynthetically active radiation average and maximum ozone concentrations.

Month	Rain-AA	Rain-OTC**	T- AA	T-OTC	PAR-OTC	RH - AA	RH OTC	[O ₃] 24H mean	Max [O ₃]
Unit	mm	mm	°C	°C	μmol m ⁻² s ⁻¹	%	%	ppb	ppb
March	21	43	12	12	277	60	64	31	57
April	89	122	15	15	331	61	66	28	61
May	90	164	17	18	309	56	65	31	62
June	151	172	22	23	393	60	65	41	105

* Only the period 28-31 March was considered

** includes rainfall and irrigation

AA refers to ambient air conditions

3.4.1.1 ozone exposure (AOT40)

Table 3.2 reports the monthly accumulated O₃ exposure (AOT40) in different ozone treatments. The AOT40 values recorded in March and April were similar in different treatments. In particular, the AOT40 value for April in the CF-OTCs is about the half compared to other treatments because the activation of OTCs and then filtration with activate carbon started in the middle of the month (17th April). The values of AOT40 in the other three treatments are similar to April because the active fumigation was started towards the end of the month, on April 23. In these two months (the most important for the crop growth) the NF treatment has accumulated an AOT40 value of about 5324 ppb.h, while the two O₃-enriched treatments have accumulated the AOT40 value of 8577 ppb.h and 21245 ppb.h in OZ+ and OZ++ respectively. In general, looking at the total values of AOT40 calculated for the whole experiment the levels in CF-OTC remained below the critical level of 3.000 ppb.h established by UN-ECE (UNECE, 2004), while in all other treatments this level has been largely exceeded by more than 2, 3 and 7 times.

Table 3. 2 - Monthly values of accumulated ozone over a threshold of 40 ppb.h (AOT40) index in CF, NF, OZ+ and OZ++ Open-Top Chambers.

Month	CF OTC (ppb. h)	NF OTC (ppb. h)	OZ+ OTC (ppb. h)	OZ++ OTC (ppb.h)
March*	159	198	155	120
April	570	1058	1200	1353
May	23	1589	3852	11672
June	368	3735	4725	9573
Total	1120	6580	9932	22718

* refers to the 4 last day of month

Table 3. 3 - Ozone exposure (AOT40) related to the different phenological stages of durum wheat. AOT40 values was cumulated from the beginning of each phenological stage to the beginning of the following stage. CF-OTC: Charcoal-Filtered Open-Top Chambers; NF-OTC: ambient O₃ concentration; OZ+ OTC: NF implemented by 30% ozone; OZ++ OTC: NF implemented by 60% ozone.

Phenological stages	COLOMBO					SCULPTUR				
	Start date	CF ppb.h	NF ppb.h	OZ+ ppb.h	OZ++ ppb.h	Start date	CF ppb.h	NF ppb.h	OZ+ ppb.h	OZ++ ppb.h
Sowing	03 March	0	0	0	0	03 March	0	0	0	0
Plant emergence	13 March	0	0	0	0	13 March	0	0	0	0
Early tillering	10 April	337	437	317	239	10 April	337	437	317	239
Plant rising	19 April	391	436	368	299	19 April	391	436	368	299
Flag leaf	05 May	1	603	1274	1999	02 May	1	424	765	1101
Earing	15 May	1	426	1224	3705	11 May	0	490	1307	3229
Anthesis	20 May	3	400	841	2406	17 May	1	179	685	2245
Harvest	28 June	387	4278	5908	14070	28 June	390	4614	6490	15605

Table 3.3 reports the plant ozone exposure (AOT40) from the starting to the end of each principal phonological stage. Both cultivars were grown in the same OTC conditions and exposed to the same ozone levels. The **table 3.3** shows that the phenological development of the two cultivars was similar from sowing to plant rising (no difference in the start date of these phenological stages). However flag leaf emergence, earing and anthesis occurred in Sculptur about three days earlier than Colombo.

The hourly mean ozone concentration accumulated over a threshold ozone concentration of 40 ppb during daylight hours (AOT40), at the end of the experiment was 22718 ppb.h , 9932 ppb, 6580 ppb and 1120 ppb in the OZ++, OZ+, NF, and CF Open-Top Chambers respectively (**Figure 3.1**). The AOT40 in the filtered CF-OTCs did not exceed the critical level value of 3000 ppb.h established for agricultural crops phytotoxicity (CLRTAP, 2010). However, this value was already reached on 6th , 13 and 30th May for plants grown in OZ++, OZ+ and NF treatments respectively.

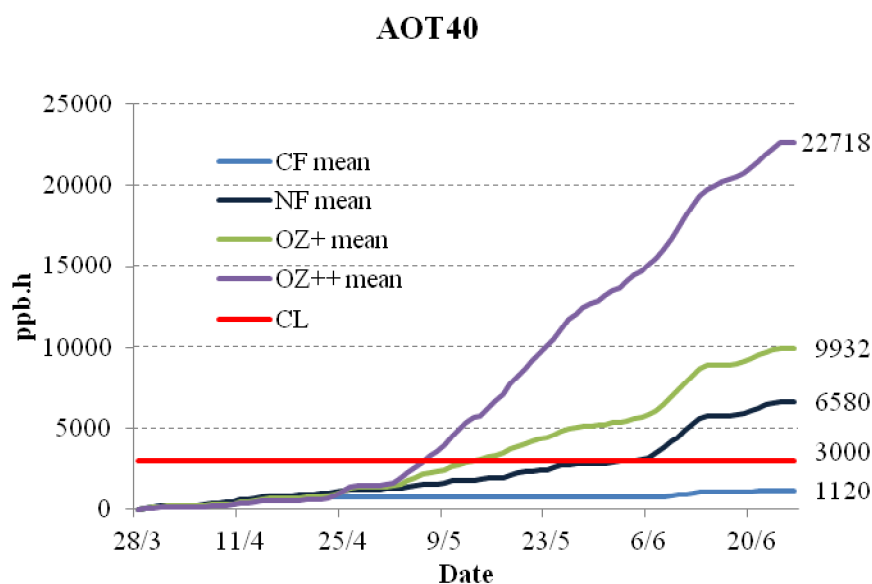


Figure 3. 1- Seasonal exposure (AOT40) of two durum wheat cultivars subjected to four levels of ozone treatments during daylight hours. The blue line indicates the exposure in the filtered Open-Top Chambers (CF). The black line indicates the non filtered Open-Top Chambers (NF). The green and purple and lines indicate the exposure to 30% (OZ+) and 60% (OZ++) respectively compared to NF. The red line shows the critical level of ozone exposure set by European legislation (3000 ppb.h).

3.4.2 Stomatal conductance

Figure 3.2 and **Table 3.4** report the effects of ozone and chitosan separately and in combination on stomatal conductance. The statistical analysis conducted on all measurements, showed that ozone reduced significantly the stomatal conductance in both cultivars (-27% **, Colombo; -37% *, Sculptur). Even treatment with only chitosan significantly reduces the stomatal conductance (gs) in both cultivars (-29% *** Colombo, -35% *** Sculptur). The cross stresses do not show significant effects, it is likely that the overlap of the two stresses causes a greater variability of response in the population of treated plants that show, however, a total decrease of stomatal conductance (gs) however not significant (**Table 3.5**). Chitosan does not seem to have any effect on Colombo since the difference between CF and OZ ++ does not seem to change in the presence of chitosan(**Figure 3.2 C**). In Sculptur instead there is a slight decrease of the difference between CF and OZ ++ when added chitosan (**Figure 3.2 D**). The stomatal conductance values used in the **Figures 3.2A,B,C,D** refer to the means of four days measurements as reported in materials and methods.

Figures 3.2E,F report the seasonal evolution of ozone effects on stomatal conductance, showing that this parameter started to be negatively affected after 14th May in both durum wheat cultivars.

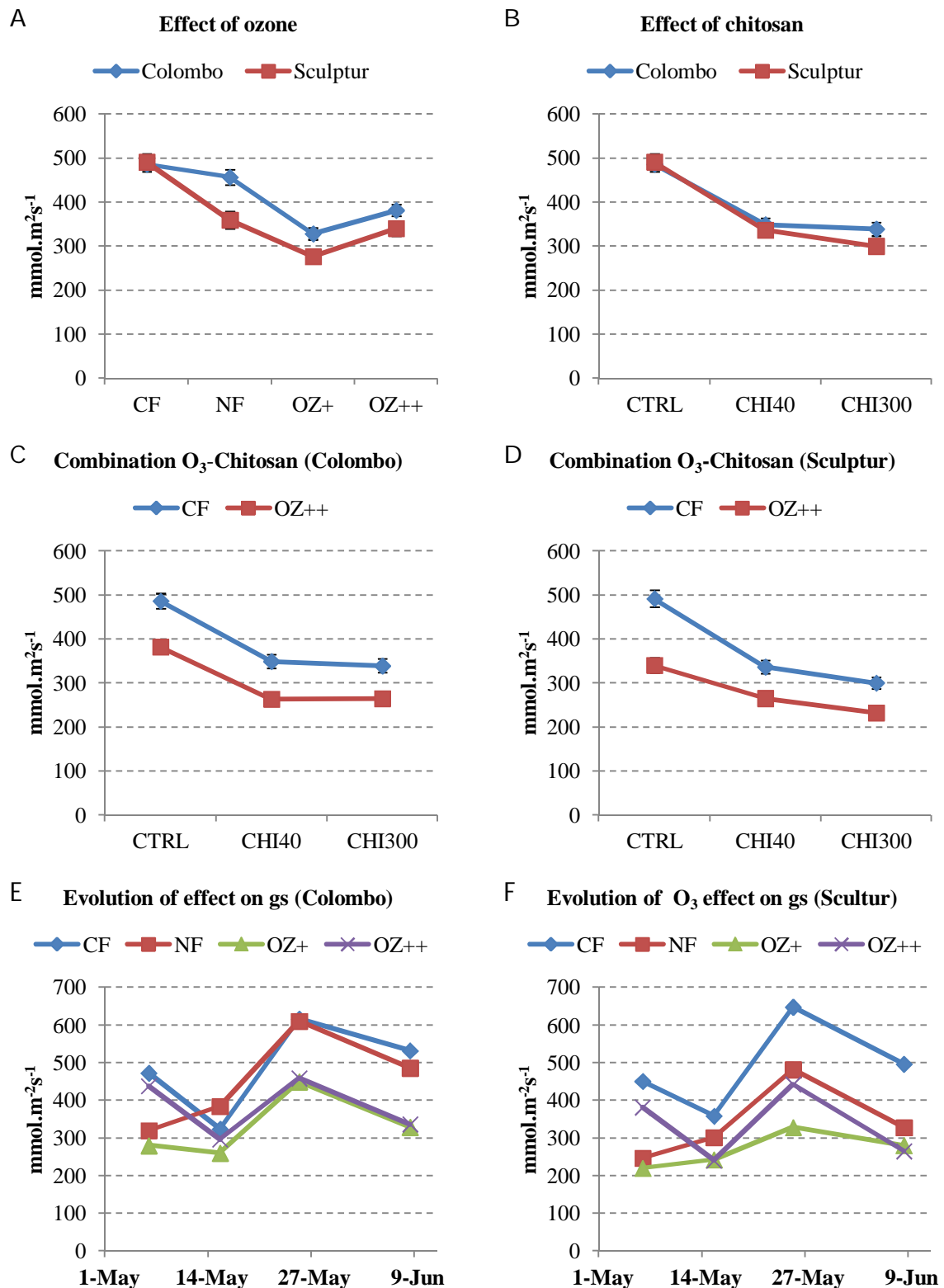


Figure 3. 2- Effects of ozone exposure (A), chitosan treatment (B) and their combination (C,D) on stomatal conductance for plants of two durum wheat cultivars grown in OTCs and the evolution of ozone effects on the stomatal conductance (E,F) during the 2015 growing season.

Table 3. 4 - Effects of ozone exposure and chitosan treatment on stomatal conductance. CF=charcoal OTC, NF: ambient ozone concentration, OZ+= NF concentration implemented by 30% ozone, OZ++=NF concentration implemented by 60% ozone, CTRL=control plants, CHI40=plants treated with chitosan 40 kDa, CHI300= plants treated with chitosan 300 kDa, St Err= standard error.

Treatments	COLOMBO		SCULPTUR	
	Mean	St. Err	Mean	St. Err
Effect of ozone				
CF	485.8	±17.1	491.2	±19.1
NF	456.6	±17.2	359.2	±19.6
OZ+	328.0	±13.2	276.3	±13.2
OZ++	381.3	±13.2	340.1	±17.2
Effect of chitosan				
CTRL	485.8	±17.1	491.2	±19.1
CHI40	348.7	±15.1	336.1	±15.3
CHI300	338.7	±15.5	299.8	±13.1
Combined effect of O ₃ and Chitosan				
O ₃ *CHI40	263.3	±12.0	264.7	±13.9
O ₃ *CHI300	264.2	±10.7	232.0	±13.1
O ₃ *CHI	263.8	±8.0	248.4	±9.6

Table 3. 5 - Statistical significance of ozone exposure and chitosan treatment on stomatal conductance. Difference between treatments is significant for * p≤0.05, ** p≤0.01 and * p≤0.001. ns=non significant difference.**

Treatments	COLOMBO		SCULPTUR	
	<i>p</i>	<i>Significance</i>	<i>p</i>	<i>Significance</i>
Ozone	0.0015	**	0.0375	*
Chitosan	0.0000	***	0.0000	***
O ₃ *CHI	0.7472	ns	0.1430	ns

The effects of ozone and chitosan on net photosynthesis (net assimilation) are reported in the **Figure 3.3** and **Table 3.6**. Ozone treatment alone caused a significant decrease in net assimilation (net photosynthesis) in Sculptur (-13.8% *), this reduction was more remarkable in Colombo (-21%) but not statistically significant, although very close to the limit of significance ($p=0.056$) This uncertainty is possible due to the higher degree of variability in the values recorded for this parameter. Nonetheless, the above data show a clear negative effect of ozone exposure on photosynthesis in both cultivars.

Chitosan treatment alone induced negative effect on net photosynthesis, but this effect was weaker than ozone and always not significant (**Table 3.7**). The combined stresses showed a tendency to additivity of the two single effects, but, again, no statistical significance was found, due to the high variability in the data.

Regarding the parameters of the curves response of assimilation to CO₂ concentration conducted only on the plants from CF and OZ ++ treatments (not subjected to chitosan), it is highlighted in Colombo a significant negative effect of ozone on the Amax parameter (-14% *), mesophyll conductance (-18% *) and TPU (-20.8% *). While the negative effects

of V_{cmax} , R_d and J despite its intensity were not statistically significant. No significant effect was observed in Sculptur. **Figure 3.4** indicates the effects of ozone on the maximum rate of Rubisco carboxylation (V_{cmax}), maximum rate of photosynthetic transport (J), triose phosphate use (TPU) and mitochondrial respiration in light (R_d^*) for both cultivars Colombo and Sculptur. The maximum assimilation (A_{max}) is reported in the **Figure 3.5**.

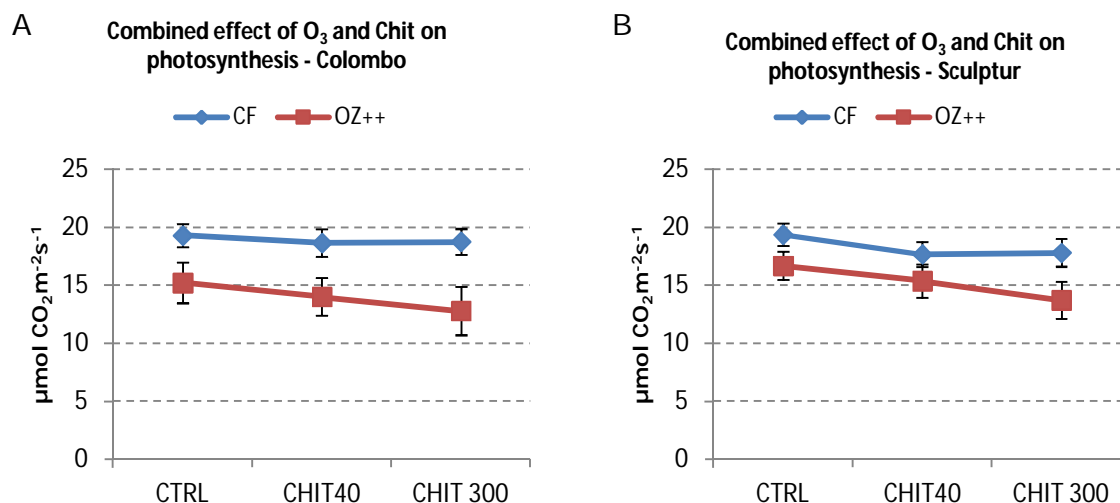


Figure 3.3- Effects of ozone and chitosan on net photosynthesis in Colombo cv (A) and Sculptur cv (B).

Table 3.6 - Effects of ozone exposure and chitosan treatment on net photosynthesis (Pn). CF=charcoal OTC, OZ++=ambient ozone concentration implemented by 60%, CTRL=control plants, CHI40=plants treated with chitosan 40 KDa, CHI300= plants treated with chitosan 300 KDa, St Err= standard error.

Treatments	COLOMBO		SCULPTUR	
	Mean	St Err	Mean	St Err
Effect of ozone				
CF	19.27	± 1.00	19.34	± 0.96
OZ++	15.19	± 1.75	16.67	± 1.21
Effect of chitosan				
CTRL	19.27	± 1.00	19.34	± 0.96
CHI40	18.64	± 1.20	17.66	± 1.07
CHI300	18.74	± 1.11	17.79	± 1.20
Combined effects of O_3 and chitosan				
O_3 *CHI40	13.99	± 1.62	15.36	± 1.45
O_3 *CHI300	12.78	± 2.09	13.70	± 1.58
O_3 *CHI	13.39	± 1.31	14.53	± 1.07

Ozone effect on net photosynthesis was calculated only for plants CTRL (no chitosan treatment). Effect of chitosan on net photosynthesis was estimated considering only plants in CF-OTCs. The combined effects of ozone and chitosan on this parameter were evaluated on the plants grown in the OZ++ OTCs (O_3 -enriched OTCs) and treated with chitosan.

Table 3. 7 - Statistical analysis of ozone and chitosan effects on net photosynthesis for two durum wheat cultivars. Difference between treatments is significant for * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. ns=non significant difference.

Treatment	COLOMBO		SCULPTUR	
	<i>p</i>	Significance	<i>p</i>	Significance
Ozone	0.05648	ns	0.01433	*
Chitosan	0.70143	ns	0.39222	ns
Ozone*chitosan	0.93460	ns	0.85204	ns

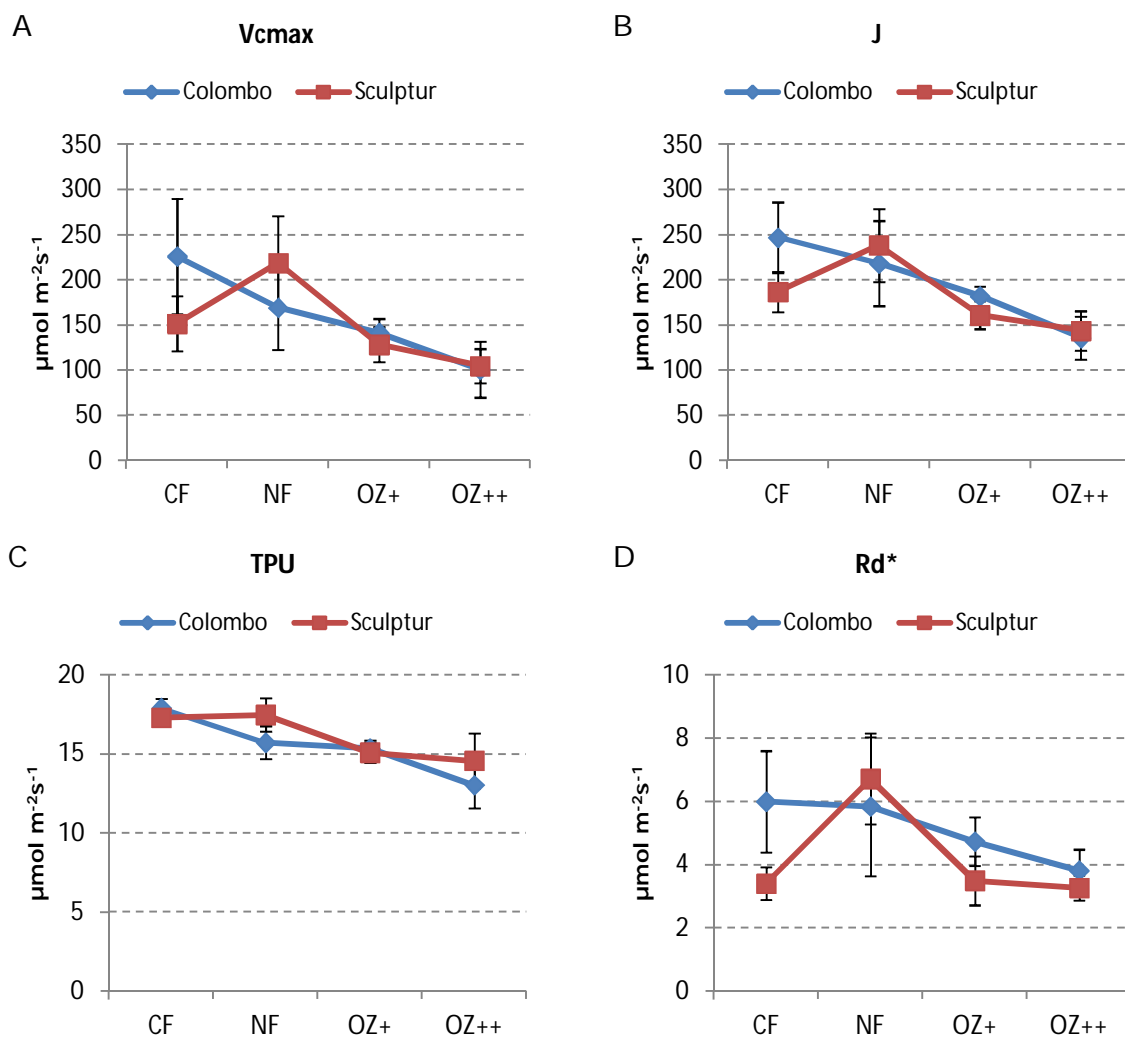


Figure 3. 4- Effects of ozone on the maximum rate of Rubisco carboxylation (V_{cmax} (A)), the maximum rate of photosynthetic transport (J (B)), the triose phosphate use (TPU (C)) and the mitochondrial respiration in light (R_d^* (D)) in two cultivars of durum wheat.

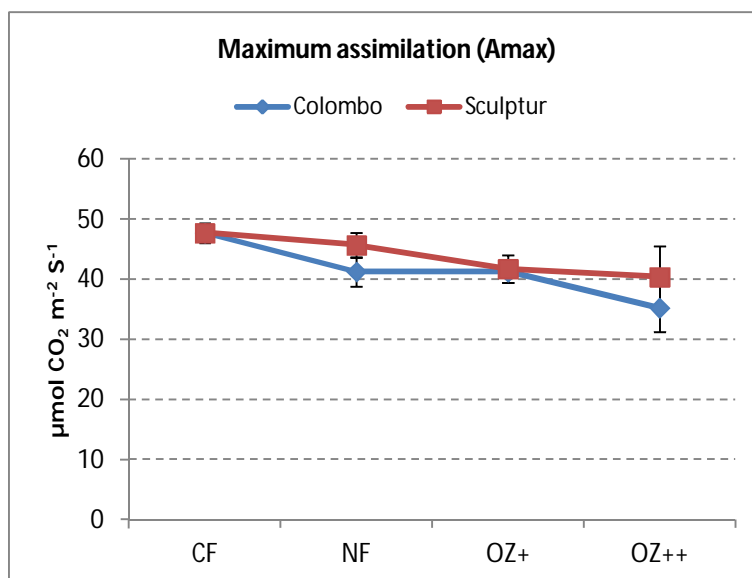


Figure 3. 5- Effects of ozone on the maximum assimilation of CO₂ (Amax) in two durum wheat cultivars (Colombo and Sculptur).

3.4.3 Chlorophyll fluorescence

Figure 3.6 and Table 3.8 indicate the effects of ozone and chitosan separately and in combination on chlorophyll fluorescence for two durum wheat cultivars.

Ozone effect on the chlorophyll fluorescence (expressed as PIabs) was found significantly different only in Colombo (-20% *), while a reduction of the same amount was not significant in Sculptur (Table 3.9). More than 50% of the ozone effects on the chlorophyll fluorescence was observed in the data of measurements carried out on May 30th for both cultivars (Figure 3.6). Even treatment with chitosan and the cross effect cause an overall reduction of PIabs, although not significant in both cases and in both cultivars. There is tendency to an additivity of the effects of both factors (ozone and chitosan) but not statistically significant.

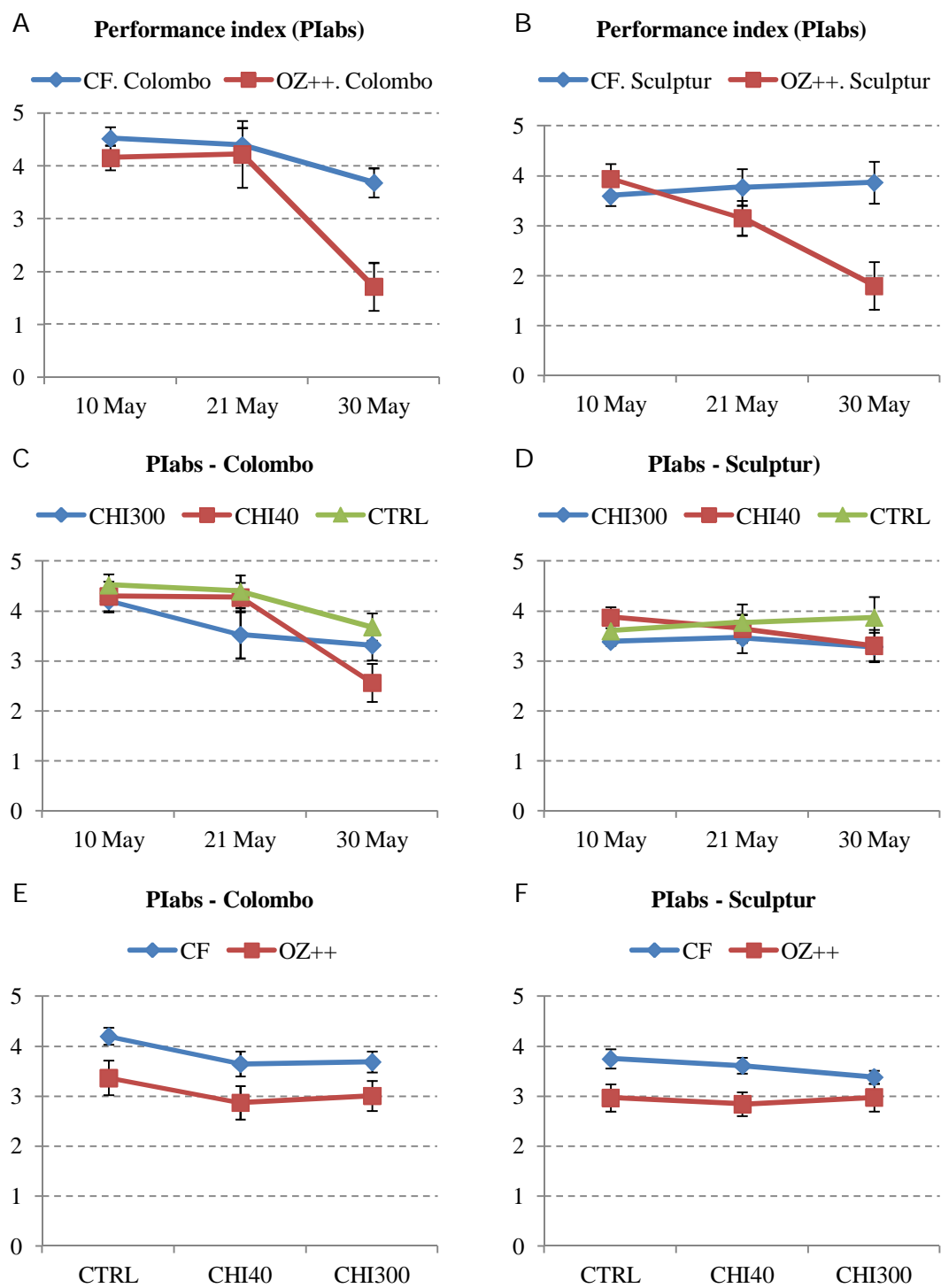


Figure 3. 6- Effect of ozone (A,B) and chitosan (C,D) and their interaction (E,F) on chlorophyll fluorescence expressed as performance index (PIabs).

Table 3. 8 - Effect of ozone exposure and chitosan treatment on Performance Index (PIabs). CF=charcoal OTC, OZ++=ambient ozone concentration implemented by 60% ozone, CTRL=control plants, CHI40=plants treated with chitosan 40 KDa, CHI300= plants treated with chitosan 300 KDa, St Err= standard error.

Treatment	COLOMBO		SCULPTUR	
	Mean	ST. Err	Mean	ST. Err
Ozone effect				
CF	4.20	±0.17	3.75	±0.19
OZ++	3.37	±0.35	2.97	±0.27
Chitosan effect				
CTRL	4.20	±0.17	3.75	±0.19
CHI40	3.65	±0.25	3.61	±0.16
CHI300	3.69	±0.21	3.38	±0.13
Mean CHI	3.67	±0.16	3.50	±0.10
Combined effects of O₃ and Chitosan				
O ₃ *CHI40	2.87	±0.33	2.84	±0.24
O ₃ *CHI300	3.01	±0.30	2.98	±0.28
O ₃ *CHI	2.94	±0.22	2.91	±0.18

Ozone effect on net performance index (PIabs) was calculated only for plants CTRL (no chitosan treatment). Effect of chitosan on performance index (PIabs) was estimated considering only plants in CF-OTCs. The combined effects of ozone and chitosan on this parameter were evaluated on the plants grown in the OZ++ OTCs (O₃-enriched OTCs) and treated with chitosan.

Table 3. 9 - Statistical analysis of ozone and chitosan effects on performance index (PIabs) for two durum wheat cultivars. Difference between treatments is significant for * p≤0.05, ** p≤0.01 and * p≤0.001. ns=non significant difference.**

Treatments	COLOMBO		SCULPTUR	
	p	Significance	p	Significance
Ozone	0.0331	*	0.0906	ns
Chitosan	0.4300	ns	0.8195	ns
O ₃ *Chitosan	0.9725	ns	0.7692	ns

3.4.4 Leaf visible symptoms

Small yellow spots began to appear on leaf surface of Colombo plant grown in OTCs OZ++ soon after AOT40 overcame 3000 ppb.h the first week of May (**Figure 3.7a**). These spots became more numerous in the following weeks, often degenerating in necrotic lesions (**Figure 3.7g**). A similar symptomatology, though to a lesser extent, was observed in the same plants treated with chitosan and exposed to OZ++ (**Figures 3.7b,h**). No symptoms appeared in Colombo plants grown in OTCs CF (**Figure 3.7c**), with the exception of a few chlorotic spots present after anthesis and later on (**Figure 3.7i**). Furthermore, these control plants did not undergo premature senescence as those from ozonated OTCs. Sculptur plants showed to be more tolerant than Colombo in terms of

visible symptoms that appeared in a milder form only late in the season, just before the beginning of leaf senescence in the middle of June. In fact, at both sampling dates in May all Sculptur plants were symptomless, including those grown in OTCs OZ++ , independently from chitosan treatment or not (**Figure 3.7d,e,f,l,m,n**). However, as for Colombo also in ozone-exposed Sculptur plants leaf senescence was anticipate by a week in respect to control plants growing in OTCs CF.

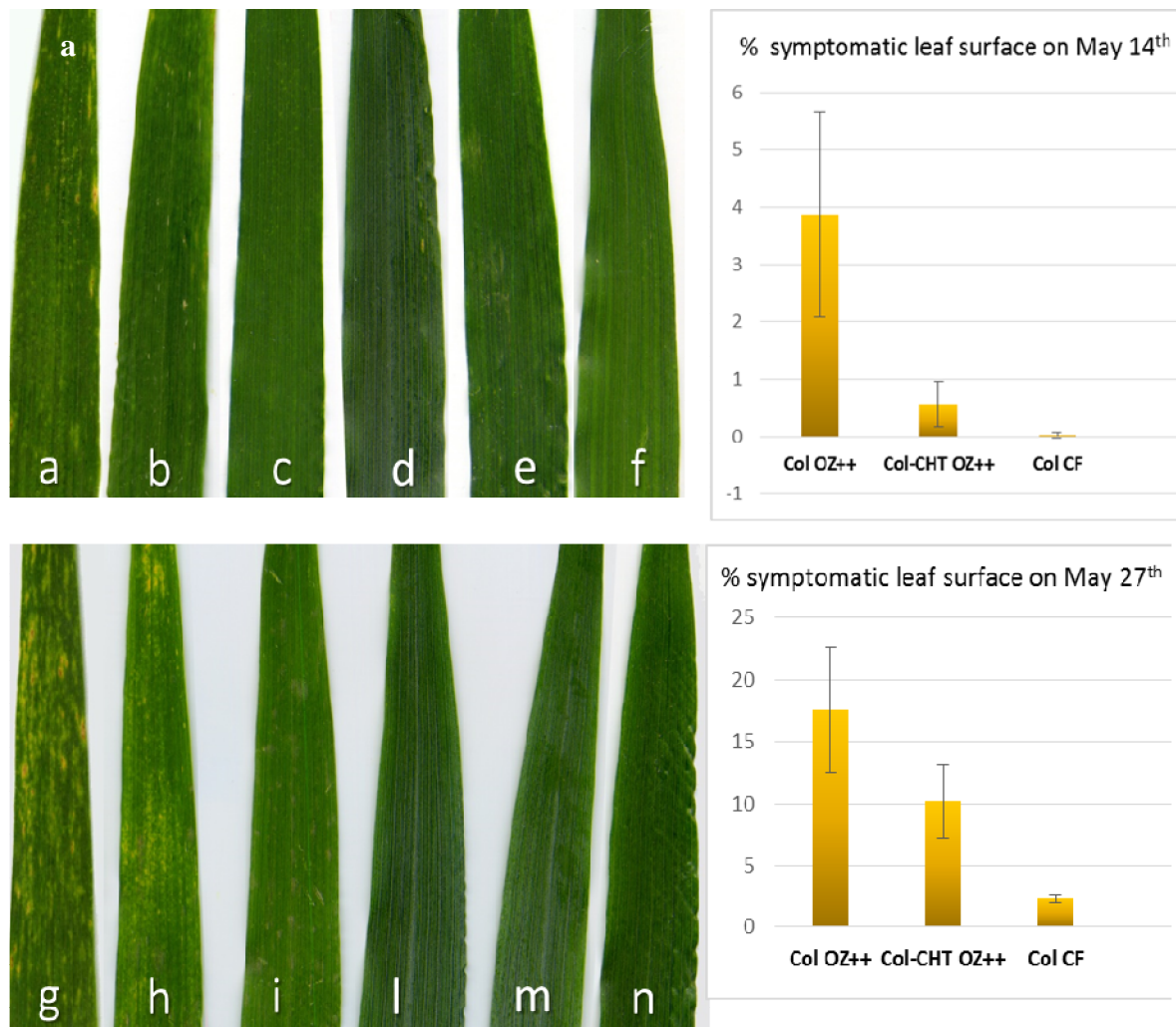


Figure 3. 7 - Analysis of ozone-like symptoms on the flag leaves of two durum wheat cultivars (Sculptur and Colombo) on May 14th (at full expansion of flag leaves) and on May 27th (after anthesis). In cv Colombo from OTCs OZ++ some chlorotic spots were already presents on May 14th (a) and became more numerous and often necrotic on May 27th (g). Colombo plants treated with chitosan (CHT) and grown in the same OTCs OZ++ were less symptomatic at both sampling dates (b,h), though not significantly on May 27th. A few chlorotic spots appeared also in Colombo grown in OTCs NF at the second sampling date (i) but not on May 14th (c). No visible symptoms were detectable in Sculptur from OTCs OZ++ (d,l) also from plant treated with CHT (e,m), nor from OTCs CF (f,n). The % of symptomatic leaf surface is referred to a square cm of leaf lamina and has been calculated as reported in materials and methods. Col OZ++: Colombo plants exposed to ozone only; Col-CHT OZ++: Colombo plants treated with both chitosan and ozone; Col CF: Colombo plants grown in filtered OTCs without chitosan treatment.

3.4.5 Crop productivity (yield)

Table 3. 10 - Statistical significance of ozone and chitosan on the harvest parameters in two durum wheat cultivars (Colombo and Sculptur). Difference between treatments is significant for * $p \leq 0.05$, ** $p \leq 0.01$ and * $p \leq 0.001$.**

Parameters	COLOMBO			SCULPTUR		
	O ₃	Chitosan	O ₃ *chitosan	O ₃	Chitosan	O ₃ *chitosan
	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>
Plant height	0.9854	0.3074	0.1570	0.0602	0.0051**	0.0181*
Total grain yield	0.0180*	0.4089	0.7819	0.0714	0.0906	0.0294*
Tot number of spikes	0.0293*	0.3127	0.3511	0.0642	0.0165*	0.0104*
Tot Abg. Biomass	0.0879	0.5094	0.2898	0.1421	0.0274*	0.0042***
Dry weight of Spikes	0.0318*	0.4512	0.3316	0.1063	0.2122	0.0251*
Dry weight of Stems	0.1894	0.3937	0.3196	0.2068	0.1261	0.1610
Harvest Index	0.2111	0.6770	0.6292	0.0089**	0.9031	0.1585
Hectolitre weight	0.0837	0.1636	0.6490	0.0191*	0.1690	0.4404

Table 3. 11 - Percentage effect of ozone and chitosan treatment on two durum wheat cultivars (Colombo and Sculptur).

	Unit	COLOMBO				SCULPTUR			
		CF	O ₃	CHI	O ₃ *CHI	CF	O ₃	CHI	O ₃ *CHI
Plant height	Cm	76 ±2.0	-1.8%	-2.9%	-0.4%	69±1.6	+1.9%	-0.5%	+0.7%
Tot G yield	g	311±44	-16%	+1.3%	-16.9%	323±42.2	-9.8%	+4.0%	-21%
Tot spikes N°	n°	27±2.2	+5%	+3.9%	+5.6%	25.4±1.2	+8.8%	+2.5%	+13.2%
Abg. Biomass	g	123±21	-14.6%	+3.0%	-17.7%	113.1±11	-0.5%	+2.1%	-10.4%
DW of Spikes	g	76±11.1	-18.6%	-2.2%	-18.3%	73.8±9.1	-6.7%	+2.4%	-16.3%
DW of Stems	g	48±11.5	-8.1%	+11.2%	-14.9%	39.3±3.4	+11.2%	+1.4%	+0.6%
H. index	%	0.42±0.0	-2.1%	-1.3%	+0.2%	0.48±0.0	-9.4%	+1.6%	-12.2%
Hect. weight	Kg/L	81.1±0.7	-2.24%	+0.6%	-2.1%	78.87±0.6	-4.1%	-0.2%	-5.5%

Ozone effect alone was calculated using the OZ++ and CF treatment for making possible the comparison of the responses of these two cultivars between the previous experiment (2013) and the present (2014). The combined effects of ozone and chitosan were calculated considering the mean of CHI40 and CHI300 in the OZ++ treatment.

Ozone exposure caused a significant reduction in grain yield (-16%, $p=0.018$) and spikes dry weight (-18.6%, *) in Colombo, whereas the number of spikes were increased (+5%, *) in presence of ozone (Table 3.10 and 3.11). Instead, in Sculptur, ozone-induced grain yield reduction was quasi-significant ($p=0.0714$, ns) leading to a significant decrease in the harvest index (-9.4%, **) and hectolitre weight (-4.1%, *). The treatment with chitosan did not show any significant effect in Colombo, while it caused a significant increase of above

ground biomass and total number of spikes (+2.1%*, +2.5%*) in Sculptur (**Table 3.10**). Instead, there was a significant increase in plant height although slight in term of percentage. The interaction between ozone and chitosan did not induce any significant effect on the yield parameters in Colombo, while in Sculptur, the combined treatment (ozone-chitosan), resulted in a significant reduction in grain yield (-21%, $p=0.0293$), aboveground biomass (- 10.4% *) and dry weight of spikes (-16.3% *) whereas it resulted in a significant increase of the height (+ 0.7%, *) and the number of spikes (+ 13.2% *). The **Figure 3.8** reports the effects of ozone and chitosan separately and in combination on the plant height, grain yield and number of spikes.

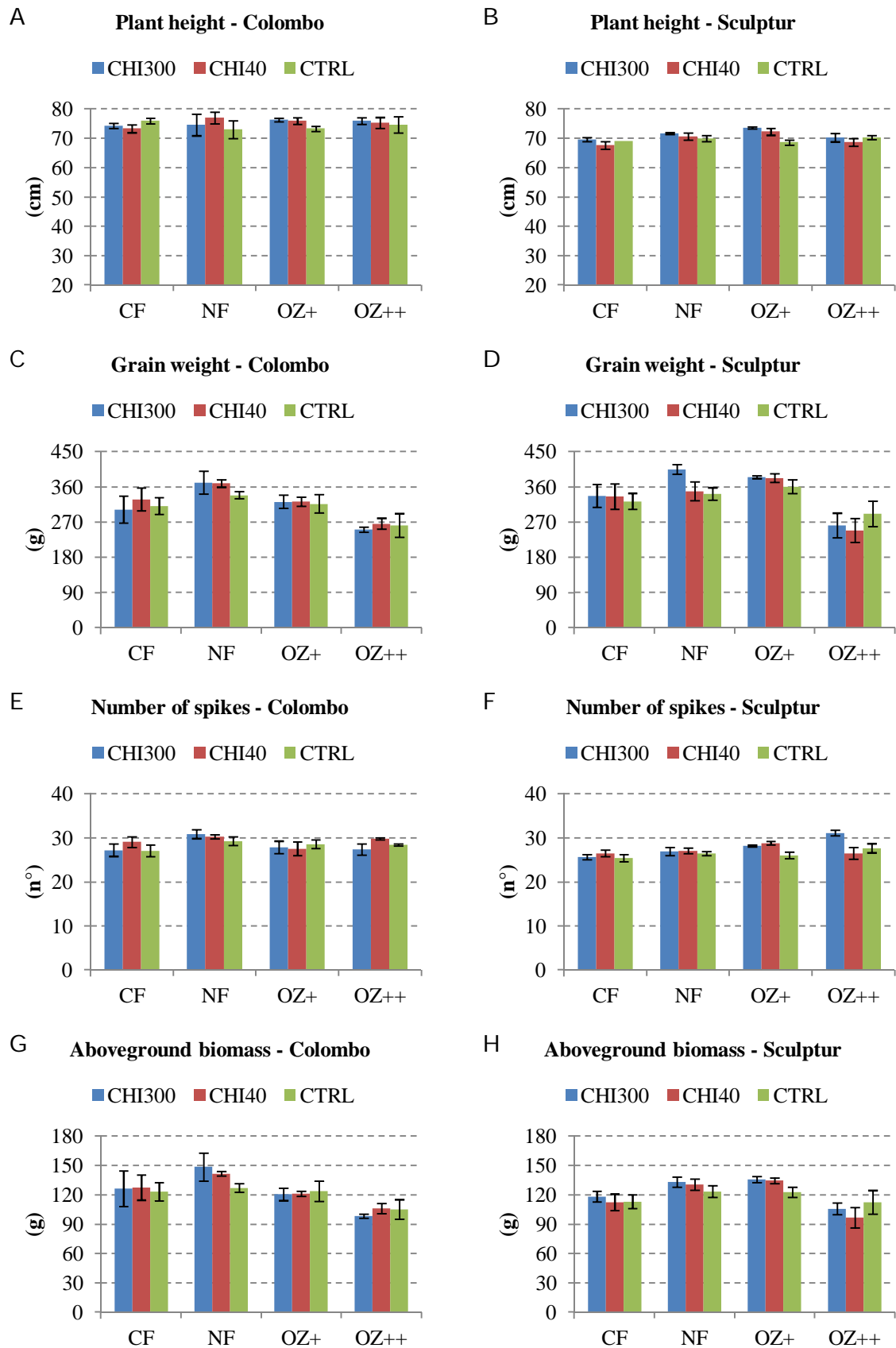


Figure 3.8- Combined effects of ozone and chitosan treatment on plant height (A, B), grain weight (C, D), number of spikes (E, F) and aboveground biomass (G, H).

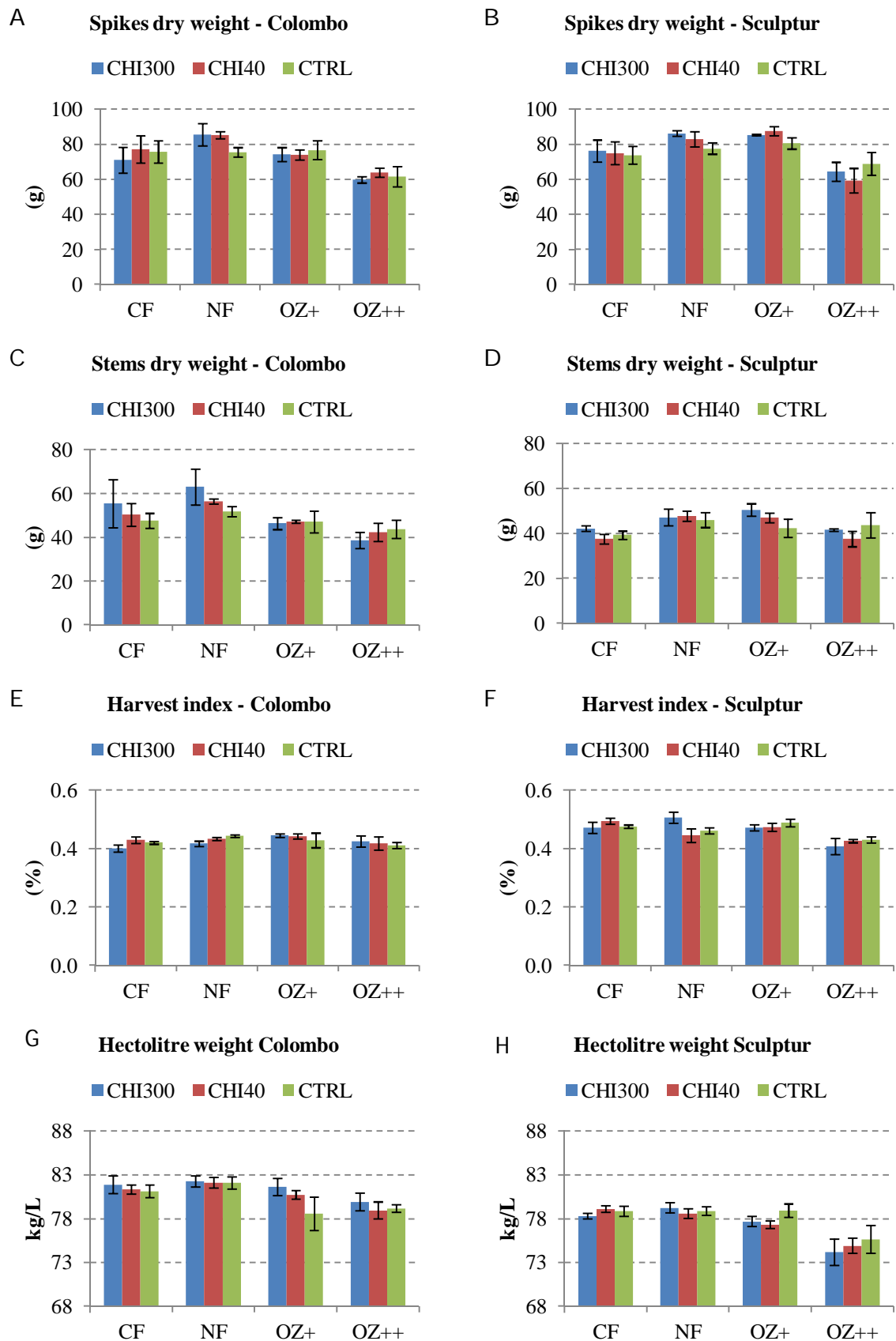


Figure 3. 9- Combined Effects of ozone exposure and chitosan treatment on spikes weight (A, B), stems weight (C, D) and harvest index (E, F) and hectolitre weight (G, H).

Tables 3.12 and 3.13 indicate the mean values of yield and yield quality parameters in different ozone and chitosan treatments for Colombo and Sculptur cultivars respectively.

Statistical analysis realized on yield parameters considering the CF treatment as control reference show that ozone alone caused significant decrease in grain yield ($p=0.0451$), harvest index ($p=0.0002$) and hectolitre weight ($p=0.0261$) regardless of cultivar. Chitosan alone did not cause any significant difference in almost yield parameters except for a decrease of the plant height ($p=0.0173$). The interaction between ozone and chitosan stimulated a significant increase of plant height ($p=0.0060$) but induced the significant reduction of aboveground biomass ($p=0.0392$) and grain yield ($p=0.0447$). The same analysis made with NF treatment as control reference report that ozone treatment alone reduced significantly the dry weight of spikes ($p=0.0337$), grain yield ($p=0.0266$), harvest index ($p=0.0020$) and hectolitre weight ($p=0.0402$). The only significant effect of chitosan treatment was the stimulation of the plant height ($p=0.0026$). The combined effects of ozone and chitosan induced a reduction of aboveground biomass ($p=0.0201$) and grain yield ($p=0.0452$).

Table 3. 12 - Mean values \pm standard error of plant height, grain yield, number of spikes, total aboveground biomass weight, spikes weight, stems weight harvest index and hectolitre weight for Colombo cultivar in different ozone and chitosan treatments.

COLOMBO		Plant height	Grain yield	Number of spikes	ABG biomass	Weight spikes	Weight of stems	Harvest index	Hectolitre weight
<i>Ozone</i>	<i>Chitosan</i>	(cm)	(g)	N°	(g)	(g)	(g)	%	kg/L
CF	CTRL	76 \pm 1.0	311 \pm 21	27 \pm 1.3	123 \pm 9	76 \pm 6	48 \pm 3	42 \pm 0	81.12 \pm 0.73
CF	CHI40	73 \pm 0.3	328 \pm 29	29 \pm 1.2	128 \pm 13	77 \pm 8	50 \pm 5	43 \pm 1	81.33 \pm 0.50
CF	CHI300	74 \pm 0.9	302 \pm 34	27 \pm 1.4	126 \pm 18	71 \pm 7	55 \pm 11	40 \pm 1	81.88 \pm 1.00
NF	CTRL	73 \pm 3.0	339 \pm 9	29 \pm 1.0	127 \pm 4	75 \pm 3	52 \pm 2	44 \pm 0	82.08 \pm 0.71
NF	CHI40	77 \pm 2.0	369 \pm 10	30 \pm 0.5	142 \pm 2	85 \pm 2	56 \pm 1	43 \pm 1	82.10 \pm 0.59
NF	CHI300	75 \pm 3.7	371 \pm 29	31 \pm 1.0	149 \pm 14	86 \pm 6	63 \pm 8	42 \pm 1	82.27 \pm 0.62
OZ+	CTRL	73 \pm 0.9	317 \pm 24	29 \pm 1.0	124 \pm 10	77 \pm 5	47 \pm 5	43 \pm 3	78.57 \pm 1.92
OZ+	CHI40	76 \pm 1.2	322 \pm 12	28 \pm 1.5	121 \pm 3	74 \pm 3	47 \pm 1	44 \pm 1	80.73 \pm 0.49
OZ+	CHI300	76 \pm 0.7	322 \pm 17	28 \pm 1.4	121 \pm 6	74 \pm 4	46 \pm 3	44 \pm 1	81.63 \pm 0.99
OZ++	CTRL	75 \pm 2.7	261 \pm 30	28 \pm 0.2	105 \pm 10	62 \pm 6	44 \pm 4	41 \pm 1	79.17 \pm 0.41
OZ++	CHI40	75 \pm 1.9	266 \pm 14	30 \pm 0.3	106 \pm 5	64 \pm 3	42 \pm 4	42 \pm 2	78.95 \pm 0.97
OZ++	CHI300	76 \pm 1.2	251 \pm 7	27 \pm 1.0	98 \pm 2	60 \pm 2	39 \pm 4	43 \pm 2	79.92 \pm 1.02

Table 3. 13 - Mean values \pm standard error of plant height, grain yield, number of spikes, total aboveground biomass weight, spikes weight, stems weight harvest index and hectolitre weight for Sculptur cultivar in different ozone and chitosan treatments.

SCULPTUR		Plant height	Grain yield	Number of spikes	A. ground biomass	Weight spikes	Weight stems	Harvest index	Hectolitre weight
<i>Ozone</i>	<i>Chitosan</i>	(cm)	(g)	<i>N°</i>	(g)	(g)	(g)	%	kg/L
CF	CTRL	69 \pm 0.0	323 \pm 21	25 \pm 0.8	113 \pm 7	74 \pm 5	39 \pm 2	48 \pm 1	78.87 \pm 0.58
CF	CHI40	68 \pm 1.3	335 \pm 32	27 \pm 0.8	112 \pm 9	75 \pm 7	37 \pm 2	49 \pm 1	79.10 \pm 0.39
CF	CHI300	70 \pm 0.7	337 \pm 29	26 \pm 0.8	118 \pm 6	76 \pm 6	42 \pm 1	47 \pm 2	78.28 \pm 0.32
NF	CTRL	70 \pm 1.0	342 \pm 16	26 \pm 0.4	124 \pm 6	78 \pm 3	46 \pm 3	46 \pm 1	78.87 \pm 0.49
NF	CHI40	71 \pm 1.2	349 \pm 24	27 \pm 0.6	131 \pm 6	83 \pm 4	48 \pm 2	45 \pm 2	78.58 \pm 0.56
NF	CHI300	72 \pm 0.3	405 \pm 13	27 \pm 0.9	133 \pm 5	86 \pm 2	47 \pm 4	51 \pm 2	79.25 \pm 0.59
OZ+	CTRL	69 \pm 0.9	360 \pm 18	26 \pm 0.7	123 \pm 5	81 \pm 3	42 \pm 4	49 \pm 1	78.93 \pm 0.75
OZ+	CHI40	72 \pm 1.2	382 \pm 11	29 \pm 0.4	135 \pm 3	88 \pm 3	47 \pm 2	47 \pm 1	77.32 \pm 0.42
OZ+	CHI300	74 \pm 0.3	385 \pm 3	28 \pm 0.2	136 \pm 3	85 \pm 0	50 \pm 3	47 \pm 1	77.68 \pm 0.57
OZ++	CTRL	70 \pm 0.7	291 \pm 33	28 \pm 1.0	113 \pm 12	69 \pm 7	44 \pm 6	43 \pm 1	75.65 \pm 1.59
OZ++	CHI40	69 \pm 1.3	249 \pm 30	27 \pm 1.3	97 \pm 10	59 \pm 7	38 \pm 3	43 \pm 1	74.92 \pm 0.88
OZ++	CHI300	70 \pm 1.5	261 \pm 31	31 \pm 0.7	106 \pm 6	64 \pm 5	42 \pm 1	41 \pm 3	74.18 \pm 1.50

3.5 Discussion

This study was focused on two durum wheat cultivars which were found sensitive to ozone in terms of visible symptoms and agronomic yield, in a previous varietal screening trial (see Chapter 2). However, in comparison to the previous experiment, this trial was planned with a more complex design, which involved 4 different levels of ozone and 2 different concentrations of chitosan application in order to investigate deeply the response of the two cultivars to ozone exposure and the role of chitosan as potential protectant for the plants against ozone damages.

3.5.1 Ozone effect

In order to evaluate the effects of the only ozone on the different parameters, we solely considered the plants belonging to the control treatment of chitosan (non-treated plants) and analyzed the differences between them.

In general, both cultivars confirmed a decrease in productivity due to the highest ozone exposure level (OZ++ treatment). Under control conditions (-50% of the ambient O₃ concentration, CF-OTCs), Sculptur proved once again to be more productive compared to Colombo (+4%), although this difference was smaller than that found in the experiment conducted in 2013. Despite the confirmation of this result, Sculptur cv in the presence of ozone did not show the same loss in grain yield observed in the previous experiment (-16%

in 2013 versus -9.8% in 2014, **Table 3.11**). Moreover, this decrease was not statistically significant (**Table 3.10**), probably because the statistical analysis were performed taking into account also the other ozone treatments (NF and OZ+), that were not applied in 2013. As a whole, the response of two durum wheat cultivars to ozone exposure appeared in some way contrasting. In Colombo a general decline of the agronomical and growth parameters was found, with grain yield and spikes dry weight showing statistically significant reductions under high ozone concentration. These results agree with the findings of Akhtar *et al.* (2010a) who reported a significant reduction of the whole-plant dry biomass and grain yield in wheat plants exposed to ozone at 60 and 100 nl l⁻¹. Ozone-induced reduction of stomatal conductance was more important in Sculptur than in Colombo (**Table 3.4**). Both cultivars showed a similar decrease in photosystem performance (PIabs) under ozone treatment even if this effect was significant only for Colombo (**Table 3.9**). The adverse effects caused by ozone exposure on grain productivity in Colombo was in correlation with stomatal conductance (gs), performance index (PIabs), net photosynthesis (Pn, ns), maximum assimilation of CO₂ (Amax), triose phosphate use (TPU). Meyer *et al.* (2000) observed a significant decrease of the net photosynthesis rate accompanied by a significant loss of grain yield in the spring wheat exposed to 65 ppb and 110 ppb for 8 hours and 4 hours daily respectively. Study of Ismail *et al.* (2014) conducted on pea plants (*Pisum sativum* L.) grown in non filtered Open-Top Chambers, suggested a significant correlation between stomatal conductance and net photosynthesis, indicating that stomatal closure could be the cause of the apparent reduction of photosynthesis.

In Sculptur, no significant effect of ozone exposure was found in all of the yield parameters except for the hectolitre weight (-4.1%, p=0.0191) and the harvest index (-9.4%, p=0.008). This latter showed a significant reduction strictly related to a grain yield reduction of the same extent (-9.8%), which resulted very close to be significant (p= 0.07). This outcome was also found in spring wheat by Pleijel *et al.* (2006), who reported a significant reduction of the grain yield and harvest index (HI) in non-filtered ozone OTCs with addition of 40 ppb of O₃. Rai *et al.* (2007) suggested a reduction of harvest index and hectolitre weight of 20.7% and 4.8% respectively for wheat grown in non filtered OTCs compared to filtered OTCs.

Moreover, the total aboveground biomass of Sculptur plants remained almost unaffected by ozone (-0.5%), indicating a clear shift in the allocation and partition of the photosynthesis products, from the grain to the stems biomass (+11.2% in OZ++ conditions,

n.s.). Regarding the physiological parameters detected in Sculptur, ozone caused a general reduction in stomatal conductance, performance index (PI_{abs}), net photosynthesis rate (P_n), maximum assimilation (A_{max}) and the maximum rate of photosynthetic transport (J), although only the difference in g_s and P_n resulted statistically significant. Previous studies reported the correlation between decline in yield and physiological parameters such as stomatal conductance (g_s), chlorophyll fluorescence, maximum assimilation of CO₂ (A_{max}), net photosynthesis rate (P_n), maximum rate of Rubisco carboxylation (V_{cmax}), maximum rate of photosynthetic transport (J) for plants subjected to ozone exposure (Rai *et al.*, 2007; Sun *et al.*, 2014; Zhang *et al.*, 2014).

The above described results show that cv Colombo is slightly more sensitive to ozone than cv Sculptur, considering grain productivity and growth parameters and visible leaf injuries. This is confirmed by opening of stomata indicating the flux of ozone in leaf tissue, the appearance of early visible ozone symptoms, leaf chlorotic lesion, plasmolysis of mesophyll cells around the substomatal cavity and swelling of chloroplasts, resulting in accelerated senescence (**Figure 3.7 a,h**). The relative tolerance of cv Sculptur to ozone is also revealed by the absence of ozone visible symptoms, and by the closure of stomata under ozone exposure resulting in a low ozone dose in the leaf tissue, unaltered mesophyll cells and consequently delayed senescence (**Figure 3.7 d,i**). Results here are consistent with the findings of Biswas *et al.* (2008b) which suggested that O₃-induced impairment of mesophyll cells in winter wheat cultivars was mainly regulated by O₃ flux, which determined the magnitude of oxidative stress.

In addition, it is important to notice that data of grain yield per both cultivars appear inconsistent with ozone gradient applied. For example grain yield was greater in the NF and OZ+ compared to CF-OTCs. However the value of this parameter was coherent with those obtained in the previous experiment.

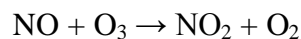
It has been observed a difference in grain yield between plants grown in filtered OTCs (CF) and those grown in non filtered OTCs (NF). Unexpectedly, plants in CF-OTCs, despite the lower ozone level, showed a lower crop yield compared to the plants in NF and OZ+ OTCs. This result leads to important differences in the assessment of the relative effect of ozone when we consider as base reference one or the other treatment. As a consequence, the assessment of the relative effect of ozone in terms of grain yield loss leads to very different results according to the different reference used for the comparison (CF or NF treatment). For example in cv Colombo the spikes number was increased by

ozone treatment compared to CF treated plants (5.2 %) but the same parameter showed a decrease in comparison to NF-OTC plants (-2.6 %). The significant grain yield reduction in cv Colombo due to ozone (-16% compared to CF, **Table 3.11**) becomes even further severe if we take the NF treatment as reference (-25%). In cv Scultpur, the grain yield decline induced by ozone exposure, although considerable in percentage terms is not statistically significant in both cases (-9.8% and -4.8% compared to CF and NF respectively).

At first sight it seems very difficult to find a possible explanation for this incoherent behavior of the agronomical parameters. The best hypothesis we can make is related to the presence of some fertilization effect on the plants belonging to the fumigated OTCs. Previous measurements of NO_x concentration (NO_x=NO+NO₂) conducted in the same facility OTCs have showed a higher concentration of NO in the CF treatments compared to the other non-filtered ozone treatments (**Figure 3.10**).

Since the total concentration of NO_x did not show any difference between CF and OZ++ chambers the increase of NO in the CF-OTCs (blue line) must be the consequence of a higher rate of conversion of NO to NO₂ in the non-filtered OTCs (OZ++ in the case of **Figure 3.10**). Similar results (increased NO and decreased NO₂ in the CF-OTC) were reported by Pleijel. (2011) and by Olszyk *et al.* (1989) even if no negative effect of NO on grain yield and biomass was observed in the plants grown in CF-treatment. Bytnerowicz *et al.* (1989) suggested also a reduction of 42% to 67% of NO₂ concentration with a simultaneous increase of NO concentrations inside charcoal-filtered chambers where ozone concentrations were reduced to 18 to 24% of the outside concentrations.

This difference in NO is likely due to the higher levels of O₃ in non-filtered and fumigated OTC (NF, OZ+, OZ++) compared to CF-OTC, and to the consequent conversion of NO to NO₂ via the following reaction:



The presence of charcoal filters (and their effect of O₃ abatement) may play a role in the effect we found on growth, as the increased dry deposition of NO₂ in the non-filtered OTCs could enhance plants fertilization and cause differences in plant growth along the growing season.

Since this “fertilization effect” seems to be absent in the OZ++ OTCs, it could be argued that in the presence of very high levels of ozone this effect is negligible and overwhelmed by the negative effect of the pollutant, while at intermediate levels of ozone the

fertilization effect is still stronger than the stress caused by the pollutant. This seems to be confirmed by the fact that NF and OZ+ treatments had quite similar AOT40 values (6580 and 9932 ppb.h respectively, **Figure 3.1 or Table 3.2**) at the end of the season, while the OZ++ treatment plants experienced much higher values AOT40 (22718 ppb.h, +345% of the NF-OTC value).

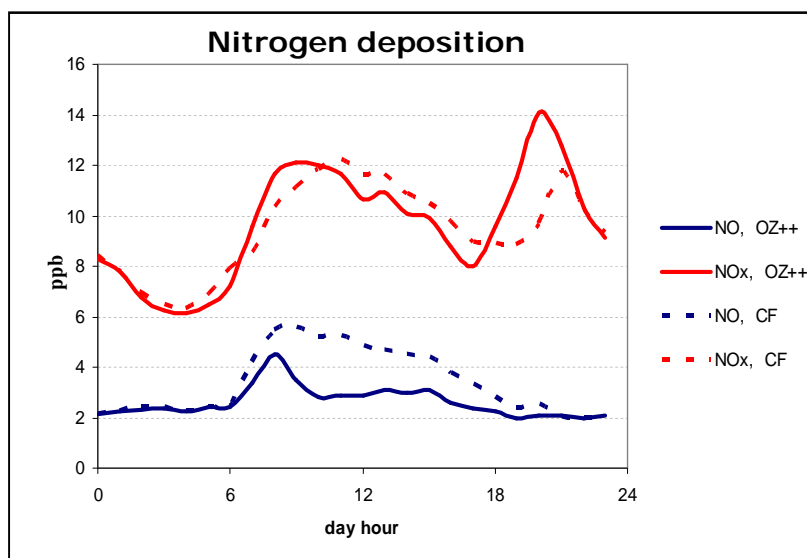


Figure 3.10- Comparison of dry deposition of nitrogen in different ozone treatments.

3.5.2 Effect of Chitosan

In this study plants reacted differently to chitosan treatment and the effect can be clearly considered cultivar dependent (see **Tables 3.10 and 3.11**). However, no significant differences were found between two chitosan types, thus we consider them here as a whole. In Colombo chitosan treatment stimulated a very slight increase of grain weight, number of spikes and total aboveground biomass compared to the untreated control plants according with other previous studies on wheat, potato, soybean and corn (Wang *et al.*, 2015; O'Herlihy *et al.*, 2003; Cabrera *et al.*, 2013; Asghari-Zakaria., 2009). Nevertheless, our results are partially in agreement with the above mentioned studies. The small (negligible) positive effect of chitosan observed in grain productivity, number of spikes and total above ground biomass seems uncoupled both from the significant reduction of stomatal conductance and from no significant reduction of chlorophyll fluorescence (PI abs) and net photosynthesis. In Sculptur the positive effect of chitosan was statistically significant for the number of spikes and total aboveground biomass but no significance was found for the difference in grain weight. Moreover this improvement could not be

explained by the other physiological parameters such as stomatal conductance, chlorophyll fluorescence and net photosynthesis, because they all decreased in plants treated with chitosan. Iriti *et al.* (2010) reported a significant improvement of pods number per plant coupled with a non significant improvement of dry weight of seeds in the case of bean treated with a 0.05% chitosan solution as leaf spraying. These findings are consistent with the present observations. Chitosan-induced stomatal conductance decrease was reported in pepper by Bittelli *et al.* (2001). The study of Del Amor *et al.* (2010) showed also a decrease of net photosynthesis and stomatal conductance for pepper plants after leaf application of other antitranspirant (proline). Chitosan induced-net photosynthesis decrease was also reported on maize and soybean one day after application (Khan *et al.*, 2002).

3.5.3 Combined effect of ozone and chitosan

The two durum wheat cultivars responded differently to combined effect of ozone and chitosan treatment. In Colombo the interaction between ozone exposure and chitosan treatment caused negligible decrease of almost all the yield parameters and this was linked to the non significant reduction of stomatal conductance, chlorophyll fluorescence (PIabs) and net photosynthesis. In Sculptur almost all yield parameters decreased in plants treated with the combination of ozone and chitosan. However, plant height and total number of Spikes increased significantly in the same treatment but not more considerable in the first parameter if the evaluation is based on percentage. The negative effect on grain yield and aboveground biomass found in Sculptur was evident only in the OZ++ plants (but not in OZ+). Considering that the ozone concentration in the OZ++ treatment is too high compared to the actual air concentration, these results are not reflecting the reality but give only the prospective situation in the case where ozone would increase in the future. This shows that chitosan could have the ability to provide a minimum protection to the plants exposed to current ambient concentration and to a moderately elevated ozone concentration but not to the higher concentrations.

The significant improvement of number of spikes reported in Sculptur is less important because wheat performance is often evaluated considering grain yield weight as main parameter. The effectiveness of chitosan has not been demonstrated perhaps by the fact that in our case plants were well watered, while chitosan application caused reduction of transpiration, breaking the natural equilibrium between water need and water consumption, resulting in overhydrated plant cells, affecting gas exchange and whole plant physiology.

The ineffectiveness of chitosan application may be also attributed to the rainfall events between two treatment campaigns, removing (washing) the chitosan-layer applied on the leaves. In the present study, chitosan treatment shows its limits in protecting plants from ozone damages because no significant positive effect is observed on grain yield (**Tables 3.10 and 3.11**) and symptoms (**Figure 3.7**) for the plants exposed to elevated ozone concentrations.

3.6 Conclusions

Both cultivars showed a significant reduction in stomatal conductance due to ozone and chitosan considering the two factors separately. However, the combination of both factors (ozone, chitosan) did not cause any significant effect. O₃-induced reduction of stomatal conductance was more determinant in Sculptur than in Colombo.

Colombo could be considered more sensitive to ozone than Sculptur referencing to (based on) ozone-like symptoms and agronomic yield. However, durum wheat sensitivity to ozone exposure seems to be complex because of intraspecific variability observed in plant response under same ozone conditions. Our four-year experiences on the durum wheat sensitivity to ozone stress have shown that same cultivar may respond differently to ozone over time. We suggest repetition of the studies on the response of a same cultivar to ozone for at least three years before confirming its sensitivity or tolerance to this pollutant. Chitosan has not proved effective in protecting plant to ozone as expected. According to stomatal conductance, chlorophyll fluorescence and net assimilation measurements, chitosan seems to play the role of oxidant instead of plant protectant from ozone stress. Difference in plant response to ozone or chitosan treatment was significantly influenced by cultivar than by other factor.

Chapter 4

The ozone-like syndrome in durum wheat (*Triticum durum*): mechanisms underlying the different symptomatological responses of two sensitive wheat cultivars

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Abstract

Colombo and Sculptur are two modern durum wheat cultivars that in previous studies proved to be very sensitive to ozone injury in terms eco-physiological parameters and yield loss. Nevertheless, their symptomatic response in terms of visible symptoms was very different, being Sculptur almost symptomless, even after several weeks of ozone exposition, whereas Colombo showed in a few weeks typical ozone-like symptoms, consisting of chlorotic spots, often degenerating in necrotic lesions. The mechanism underlying this different symptomatic responses has been studied by a biochemical and microscopical approach with the aim of verifying whether the lack of visible symptoms in Sculptur is linked to a better ROS scavenging capacity or to other mechanisms of avoidance of ozone effect, such as stomata closure, that would also explain the loss of productivity. Plants were grown in Open-Top Chambers and exposed to four different ozone concentrations: ambient concentration (NF), minus 50% (CF), plus 30% (OZ+) and 60 % (OZ++) in respect to ambient ozone level. Samples of flag leaves were collected on May 14th 2014 and on May 27th 2014 from OTCs CF and OZ++ at the stage of pre- and post-anthesis, respectively. No samples were analyzed from OTCs NF and OTCs OZ+ being the ozone effects on eco-physiological parameters and yield loss not significant between them, possibly due to the peculiar raining weather of summer 2014. Results of biochemical analyses indicated that at the first sampling date the ascorbate pool was constitutively lower in Colombo, compared to Sculptur, and that the latter was able to maintain the cellular ascorbate redox state at least up to the second sampling date, thus limiting leaf tissue injury. Furthermore, Colombo underwent a decrease in reduced glutathione content, at least up to May 14th, possibly due the scarce recycling by ascorbate, as also demonstrated by the increase in the glutathione oxidized pool. The ability of Sculptur to maintain redox homeostasis has been confirmed by microscopic observations that showed no or scarce H₂O₂ deposition in ozonate leaf tissues in comparison with the abundant H₂O₂ found in Colombo mesophyll cells around the substomatal chamber. Most of these cells were damaged or death and responsible for the appearance of chlorotic/necrotic lesions. Furthermore, in ozonate Sculptur plants much more stomata appeared closed than in Colombo, suggesting also an avoidance of ozone effect due to limited stomatal conductance. Nevertheless, transmission electron microscopy showed that in the apparent undamaged mesophyll cells of Sculptur many chloroplasts

appeared slightly swollen and with numerous plastoglobuli, as a result of a mild oxidative stress. In the light of the above observations it can be concluded that Sculptur is apparently more tolerant to ozone oxidative stress for the higher content of constitutive ascorbate pool that may scavenge ROS more efficiently than in Colombo and for the synergistic effect of stomata closure that in Sculptur seems to be maintained for a long time by the feed-back mechanisms of starch accumulation. This persistent stomatal closure and the slight damages of chloroplasts may account for the yield loss of Sculptur. Instead, the similar yield loss of Colombo is very likely attributable to the lower ROS scavenging capacity and to the less efficient stomata closure that led to severe damages of part of the mesophyll cells, however leaving a great part of this photosynthetic tissue functional.

Key words: antioxidants; glutathione; ascorbate; ozone-like syndrome; ozone-injury microscopy.

4.1 Introduction

The detrimental effect of tropospheric ozone on the vegetation in general, and on the agricultural crops in particular, has been demonstrated in a large number of studies (reviewed in Ainsworth *et al.*, 2012 and in Rai, R., Agrawal, M., 2012). This pollutant, that enters the plant leaves mainly through open stomata during normal gas exchange, produces in leaf tissues several reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl (OH^\bullet) and hydroperoxyl (HO_2) radicals (Overmyer *et al.*, 2009; Vahisalu *et al.*, 2010). ROS cause peroxidation of lipids, chlorophyll bleaching, protein oxidation, damage to nucleic acids and destruction of cell membranes, particularly those of chloroplasts (Karberg *et al.*, 2005; Vaultier and Jolivet, 2015). In response to ROS production, plants trigger a number of antioxidative stress-related defense mechanisms (Kangasjärvi *et al.*, 1994), among them: ascorbate, phenolics, α -tocopherol, glutathione, carotenoids and scavenging enzymes, i.e. superoxide dismutases, catalases and several peroxidases (Temmerman *et al.*, 2002). When ROS production overwhelms antioxidant defenses cells undergo severe damages and, ultimately, cell death. These damages may result in a number of different leaf symptoms on sensitive plants depending on the species and the exposure type. Chronic exposure, as a consequence of low ozone concentrations (< 40 ppb) for the entire life of a plant, with some episodes of high concentration (> 80 ppb), either periodically or accidentally, cause chlorosis (yellowing due to the chlorophyll breakdown, often distributed in spots over the leaf) and bronzing (red-brown pigmentation caused by phenylpropanoid accumulation) (Krupa *et al.*, 2000). Chlorosis and chlorotic spots may degenerate in necrotic lesions of different tonalities depending on the species when ozone concentrations rise over 80 ppb some days or weeks (Krupa *et al.*, 2000; Chaudhary and Agrawa, 2015). However, photosynthesis and growth can be inhibited even in absence of leaf visible symptoms when damages are restricted to the inactivation of important enzymes, such as ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco), or to the induction of stomata closure (reviewed in Iriti and Faoro, 2008). At the individual level, ozone exposure can induce significant losses of crop yields and quality (Sarkar and Agrawal, 2010; Gerosa *et al.*, 2009). For example, wheat yield losses due to ambient ozone concentrations were estimated at about 20-27% in the Mediterranean region (Fumagalli *et al.*, 2001) and at about 6-17% in the central Italy (Fagnano *et al.*, 2009).

Among wheat species *Triticum durum* is particularly sensitive to ozone damages, though this sensitivity may vary between cultivars (Gerosa *et al.*, 2014; Monga, 2015). In

previous studies we showed that two very productive cultivars, Colombo and Sculptur, were significantly damaged by the pollutant as regards eco-physiological parameters and grain yield (see chapters 2 and 3). Nevertheless, their symptomatic response in terms of visible symptoms was very different, being Sculptur almost symptomless, even after several weeks of ozone exposition, while Colombo showed in a few weeks typical ozone-like symptoms, consisting of chlorotic spots, often degenerating in necrotic lesions. The observed reduction in stomatal conductance induced by ozone in both cultivars may partially explain yield loss but not the different symptomatic response (Monga *et al.*, 2015).

To unravelling the mechanisms underlying this different response we have undertaken a biochemical and microscopical study with the aim of verifying whether the lack of visible symptoms in Sculptur is linked to a better capacity of ROS scavenging or to other mechanisms of avoidance of ozone effect, such as stomata closure, that would also explain the loss of productivity observed in this apparently tolerant wheat cultivar.

4.2 Materials and Methods

4.2.1 Plant material and ozone treatments

The experimental plan was the same as detailed in chapter 3. Durum wheat ozone-sensitive cultivars, Colombo and Sculptur were cultivated in pots maintained in *i*) OTCs CF (charcoal filtered), *ii*) OTCs NF (non filtered), *iii*) OTCs OZ+ and OTCs OZ++ non filtered supplemented with 30% and 60% of ozone, respectively. The 14th of May 2014, when the flag leaf was fully expanded and the AOT40 inside OTC OZ++ had just overcome the critical threshold of 3000 ppb.h a first collection of plant material for biochemical and microscopic study was performed. Flag leaves randomly chosen from 2 OTCs CF and 2 OTCs OZ++ were sampled and processed as described below. The 27th of the same month, soon after flowering and at the beginning of seed filling, another sampling of flag leaves was performed, using the same criterion as before. In this study no samples were analyzed from OTCs NF and OTCs OZ+ being the ozone effects on eco-physiological parameters and yield loss not significant between them, possibly due to the peculiar raining weather of summer 2014 (see chapter three). At the first sampling date flag leaves of Colombo from OTCs OZ++ already showed some chlorotic spots that become more diffuse and, in some instances, also necrotic at the second sampling date when Sculptur appeared still symptomless.

4.2.2 Biochemical analysis

Foliar sampling was done in the morning from 10 to 11 AM. A total of twenty flag leaves for each combination of cultivar and treatment, as described in the previous paragraph, were rapidly frozen in liquid nitrogen and stored at -80 °C until analysis. Three replicates were maintained for all measurements.

4.2.2.1 Ascorbic and dehydroascorbic acid determination

Frozen foliar tissue (300 mg) was ground with liquid nitrogen in a pre-cooled pestle and mortar. The powder was immediately added to 5 ml of 6% metaphosphoric acid. The homogenate was vortexed for 30 s and then centrifuged at 12,000 rpm for 15 min at 4°C.

L-ascorbic acid (AsA) was quantified by analyzing diluted aliquots of the prepared extracts by HPLC, as previously described (Picchi *et al.*, 2012). The oxidized form (dehydroascorbic acid, DHA) was determined by the “subtractive” method after measurement of the total ascorbate (AsA + DHA) content following reduction with dithiothreitol (DTT). The reduction was carried out according to Davey *et al.* (2003). Briefly, 100 µl of plant extract was added to 50 µl of a stock solution of 200 mM DTT in 400 mM Tris base. This generated a final pH of 6–6.8. The reaction was stopped after 15 min at room temperature by acidification with a further 50 µl of 8.5% orthophosphoric acid. Reduced extracts were then diluted with 0.02 M orthophosphoric acid and immediately analyzed by HPLC. The analytical column was a 250 x 6 mm i.d., Intersil ODS-3, maintained at 40 °C. The isocratic elution was performed using 0.02 M mobile phase orthophosphoric acid at a flow rate of 0.7 ml/min. Samples of 20 µl were injected and monitored at 254 nm. The identity of the AsA peak was confirmed by coelution with authentic standards and the concentration of AsA was calculated from the experimental peak area by analytical interpolation in a standard calibration curve.

4.2.2.2 Glutathione determination

Total glutathione (reduced glutathione, GSH, plus oxidized glutathione, GSSG) and oxidized glutathione were determined by the 5,5'-dithio-bis-nitrobenzoic acid (DTNB)-glutathione reductase (GR) recycling procedure (Anderson *et al.*, 1992). GSSG was reduced to GSH by the action of GR and NADPH, whereas, GSH was oxidized by DTNB to give GSSG and 5-thio-2nitrobenzene (TNB). GSSG was determined from the sample after removal of GSH by 2-vinylpyridine derivatizations. Changes in absorbance due to the rate of TNB formation were measured at 412 nm and the contents were calculated using a

standard curve. GSH was determined as the difference between total glutathione and GSSG.

4.2.3 Microscopic and ultrastructural analysis

4.2.3.1 Histochemistry

Five flag leaves for each cultivar were collected from the OTCs CF and from the OTCs OZ++ on May 14th, when visible symptoms had already started to appear in cv Colombo, and on May 27th when chlorotic lesions were clearly visible on the same cv. At both sampling date, Sculptur was still symptomless. Leaves were immediately dipped in 2-3 cm of water in 20 ml centrifuge tubes and kept in a refrigerated bag to maintain their hydration until reaching the lab. Samples (2x2 cm) were then immediately excised from the flag leaves and infiltrated with 3,3'-diaminobenzidine (DAB)-HCl to detect possible H₂O₂ accumulation sites, due to ozone-induced oxidative stress. Other leaf samples were stained with Evans blue to detect dead or damaged cells. The detailed protocols for both staining procedures can be found in Faoro and Iriti, 2005.

4.2.3.2 Light and Transmission electron microscopy

Some fragments (1-2 mm²) of the same leaves, collected on May 27th as described in the previous paragraph, were fixed in a mixture of 4% formaldehyde and 3% glutaraldehyde in phosphate buffer 0.1 M, pH 7.2, postfixed in 1% osmium tetroxide, dehydrated in an ethanol series and embedded in Spurr resin. Semithin sections were cut and stained with toluidine blue for a general overview of leaf tissues condition and to assess stomata opening. At this purpose, 2 embedded blocks for each of the 5 sampled leaf replicates from OTCs OZ++ and OTCs CF were cut in 2 µm thick serial sections and 10 of this section mounted in a microscope slide. In this way about 200 µm of tissue could be examined for each block, allowing to examine different section planes of all stomata present in the leaf tissues and to determine their aperture or closure. Observations were made with an Olympus BX50 (Olympus, Tokyo, Japan) light microscope equipped with epi-polarization filters and differential interference contrast (DIC).

Ultrathin sections were cut from representative tissue samples, based on the light microscope observations. Sections were stained with Uranyl acetate and lead citrate and examined with a Jeol 100SX TEM (Jeol, Japan).

4.2.4 Statistical analysis

The results of the biochemical analyses are expressed as means \pm standard errors (SE). Within each day of sampling data were subjected to analysis of variance (ANOVA), and comparison among means was determined according to Tukey's test. Significant differences were accepted at $P < 0.05$ and indicated with different letters. All statistical analyses were performed using the Statgraphics v.7 (Manugistic Inc., Rockville, MD, USA) software package.

4.3 Results

4.3.1 Biochemical analysis

The results of reduced ascorbate and total ascorbate contents are shown in **Figure 4.1 (a,b)**. At the two data of sampling, the cultivar Scultpur distinguished from Colombo for higher AsA and total ascorbate (AsA + DHA) contents, while the ozone treatment did not significantly affect nor the total neither the reduced form of ascorbic acid in both cultivars. In fact, the mean contents of CF and OZ++ plants were not significantly different. However in Colombo OZ++ plants a tendency towards an increase in the AsA values was recorded, particularly at the second data of sampling date (27th May), as indicated by the higher AsA/AsA+DHA ratio in respect to Colombo control plants grown in OTCs CF (**Figure 4.1c**).

As regards glutathione, on the first sampling data (14th May) the content of the reduced form did not differ in CF and OZ++ plants of both cultivars (**Figure 4.1c**). Nevertheless, the 14th May Colombo OZ++ plants had higher total glutathione content compared to Colombo CF plants (**Figure 4.1d**), as a result of an increase of the oxidized glutathione pool. This tendency determined a decrease of the GSH/(GSH+GSSG) ratio in Colombo OZ++ plants., while the highest GSH/(GSH+GSSG) ratio was observed in Scultpur OZ++ plants (**Figure 4.1f**). On the other hand, the 24th May both Colombo and Scultpur OZ++ plants showed a marked increase in the reduced glutathione content compared to their CF plants (**Figure 4.1d**). As a consequence, at the second data of sampling both the GSH+GSSG contents and the GSH/(GSH+GSSG) ratios were higher in OZ++ plants of the two cultivars (**Figure 4.1e,f**).

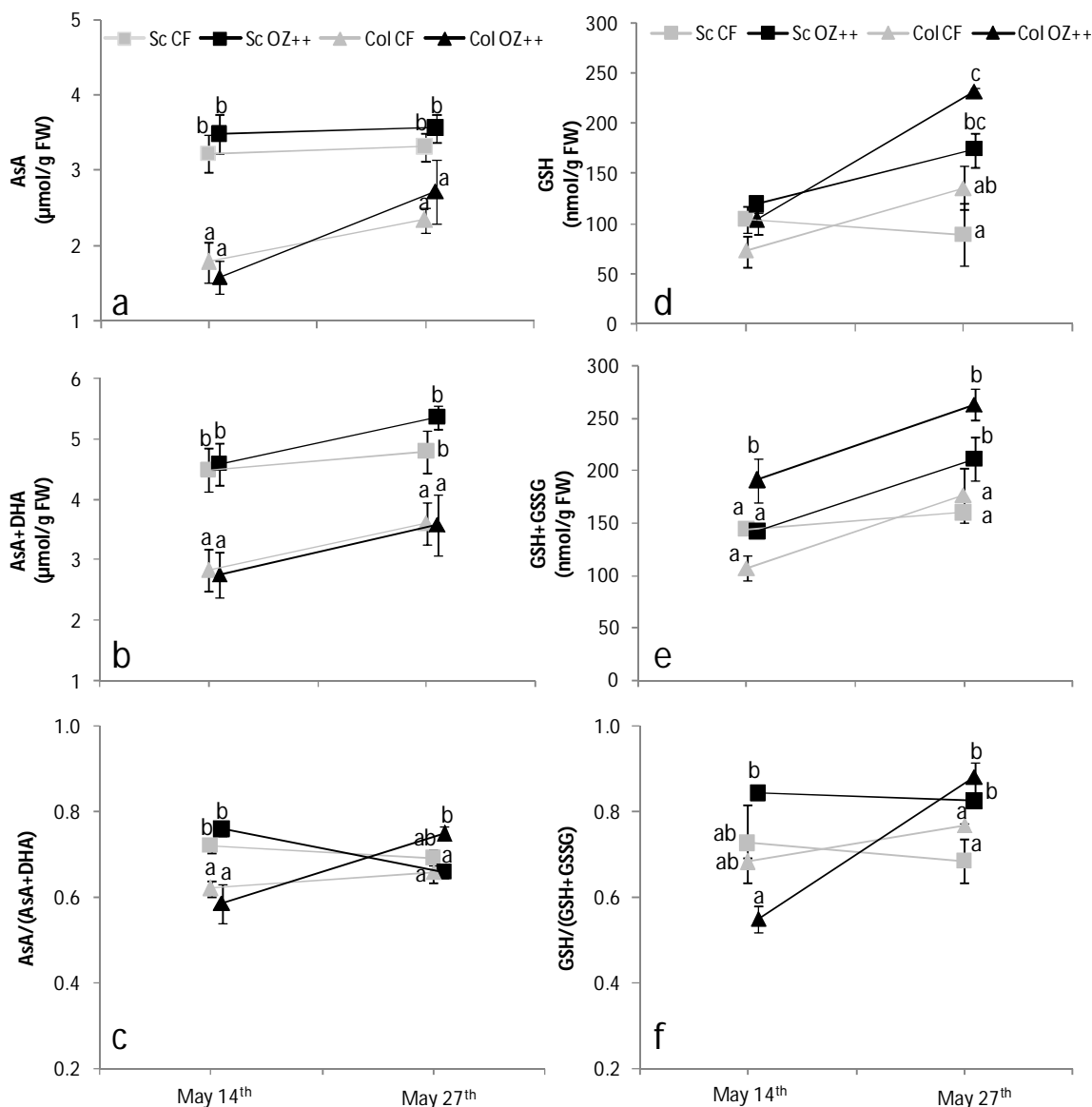


Figure 4.1– Results of contents of a) reduced ascorbate (AsA), b) total ascorbate (AsA + DHA), c) reduced ascorbate/total ascorbate (AsA/AsA+DHA), d) reduced glutathione (GSH), d) total glutathione (GSH + GSSG) and e) reduced glutathione/total glutathione (GSH/GSH+GSSG), in Sculpur and Colombo plants grown in CF or OZ++ OTCs. Vertical bars indicate SE (n=6). For each day of sampling, different letters indicate means significantly different ($P < 0.05$) according to Tukey's test.

4.3.2 Microscopic and ultrastructural analysis

In samples of Colombo flag leaves collected on May 14th, soon after AOT40 had overcome the critical threshold of 3000 ppb.h in OTCs OZ++, DAB staining of leaf fragments revealed discrete deposition of H_2O_2 in the mesophyll cells around stomata (**Figure 4.2a**). These cells appeared damaged when stained with Evans blue indicating they were undergoing cell death or were already dead (**Figure 4.2g**). No H_2O_2 deposition nor cell death were found in similar Colombo samples collected from OTCs CF (not shown) or in Sculpur leaf fragments collected from both OTCs OZ++ and CF (**Figures 4.2b,f,h,n**).

These findings match with the appearance of the first visible chlorotic spots only in Colombo flag leaves.

In samples collected on May 27th, Colombo leaf fragments showed intense H_2O_2 deposition not only in the mesophyll cells adjacent to stomata but also in the nearby tissue (**Figure 4.2c**). Most of similar cells in leaf fragments stained with Evans appeared dead, particularly those around stomata (**Figure 4.2i**), accounting for the expression of chlorotic or necrotic lesions, already visible at this stage in this cultivar. Even in Sculptur at this time lapse discrete H_2O_2 deposition was evident in mesophyll cells around stomata, though to a lesser extent (**Figure 4.2d**) and some of these cells appeared damaged or dead (**Figure 4.2l**). However, the number of damaged cells was not high enough to cause visible symptoms. No H_2O_2 deposition, nor cell death were found in both cultivars growth in OTCs CF (**Figures 4. 2e,f,m,n**) with the exception of faint H_2O_2 staining around some stomata of Colombo (**Figure 4.2e**) and related cell damages (**Figure 4.2m**) indicative of an incipient oxidative stress.

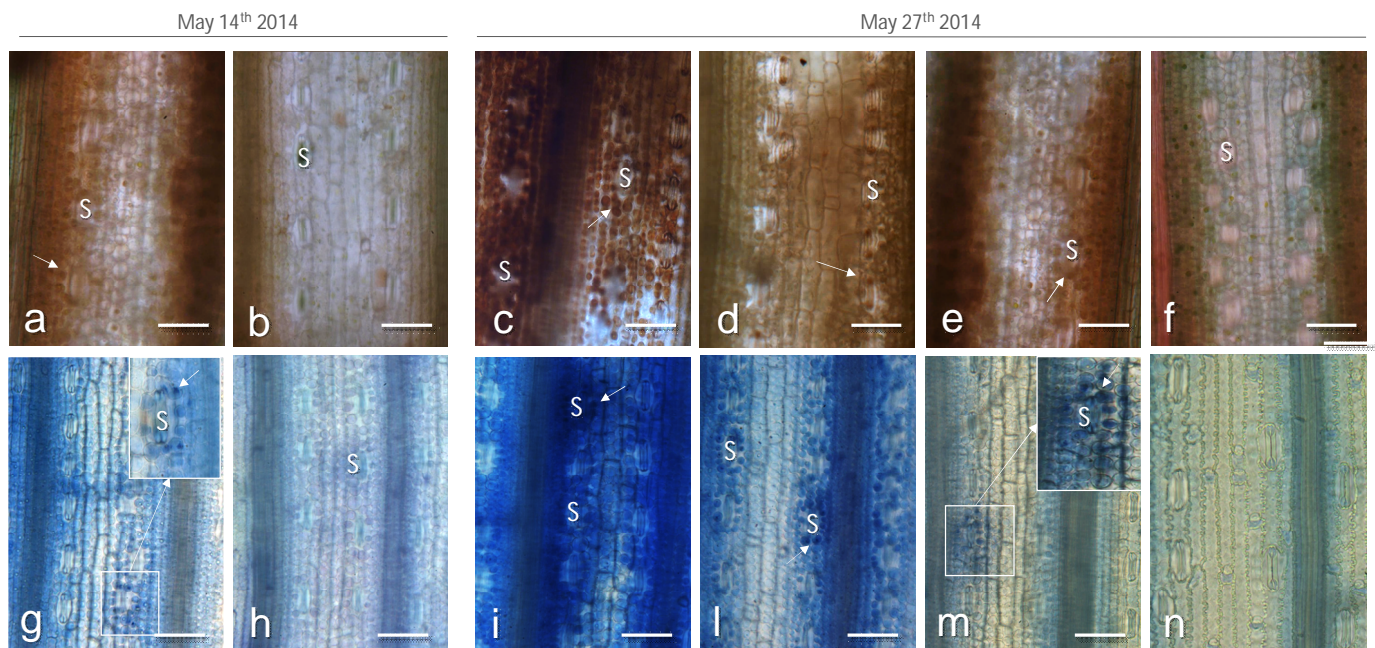


Figure 4. 2– Flag leaf fragments of durum wheat cv Colombo (a,c,e,g,i,m) and cv Sculptur (b,d,f,h,l,n) stained with DAB (a-f) for the detection of H_2O_2 (brown precipitates) and with Evans blue for cell death recognition (damaged cells in light blue, dead cell in dark blue); S, stomata; arrows point to H_2O_2 deposits or to dead cells in of the palisade tissue around stomata. Leaves in a,b,g,h were collected on May 14th from OTCs OZ++, while the others on May 27th from OTCs OZ++ (c,d, i, l) and OTCs CF (e,f,m,n). All bars=50 nm).

Semithin sections from resin embedded leaf fragments, stained with toluidine blue showed that in Colombo flag leaves from OTCs OZ++ the parenchyma mesophyll appeared plasmolysed in large areas surrounding the substomatal cavity, while other nearby tissue appear almost unaltered (**Figures 4.3a,c,e**). Cell walls were thicker in respect to control leaves grown in OTCs CF (**Figure 4.3a**) and densely stained in blue-green similarly to xylem cells, thus indicating that a lignification process was in progress (**Figure 4.3c**). Stomata did not appear particularly altered and most of them were apparently open as those from plants kept in OTCs CF (**Figure 4.3e**). Though a proper statistical evaluation was not carried out, it was evident that leaf thickness of control plants was higher than that of ozone exposed ones (**Figures 4.3a,c**), possibly due to the presence of plasmolysed cells in the latter.

No evident cell damages were observed in Sculptur leaf tissues from plants grown in OTCs OZ++ and their histology was fully comparable with control leaves from OTCs CF (**Figures 4.3b,e**). However, in ozone exposed plants stomata appeared often closed (**Figure 4.3f**). A rough estimation of closed stomata with the criterion reported in materials and methods indicated a range of closure between 40-50% in respect to 10-20% observed both in Sculptur control plants and in Colombo plants either from OTCs OZ++ or OTCs CFs.

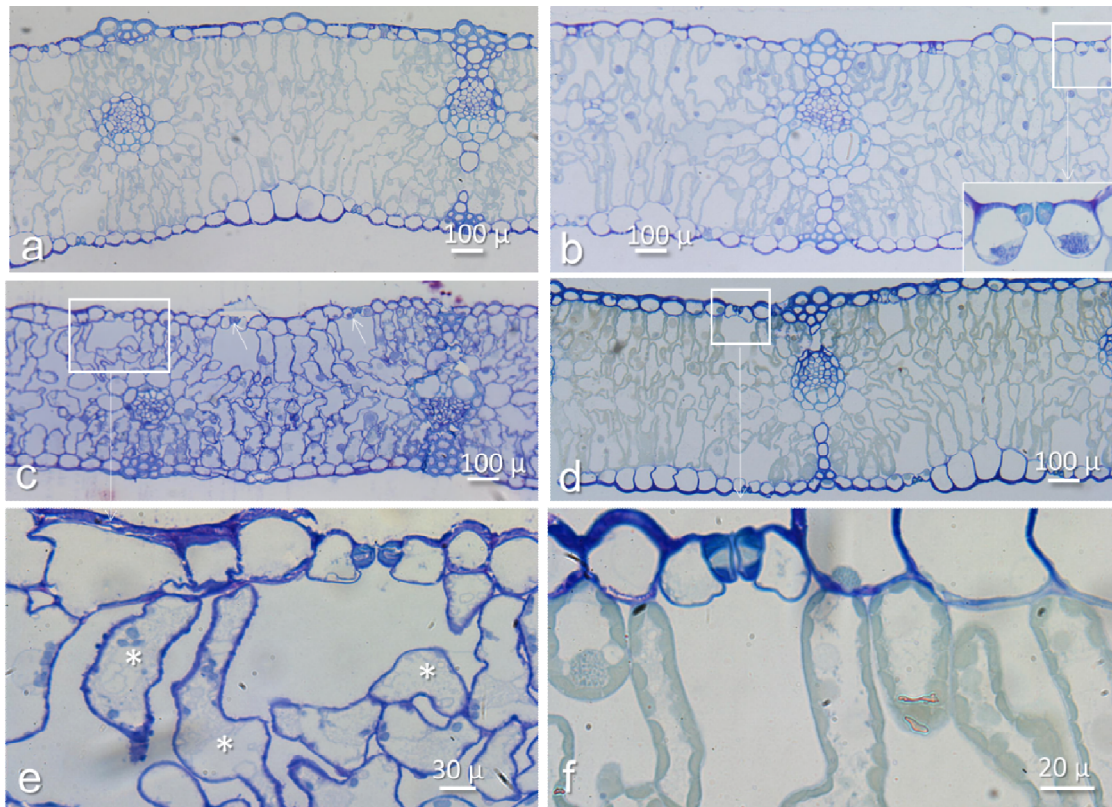


Figure 4. 3– Semithin (2 μm) cross sections, stained with toluidine blue, of flag leaf fragments of durum wheat cv Colombo (a,c,e) and cv Scultpur (b,d,f) collected on May 27th from OTCs CF (a,b) and OTCs OZ++ (c-f); e,f are enlargement of c,d, respectively. Note that many cells in ozonate Colombo leaf tissues around the substomatal cavity are plasmolysed (c,e, asterisks) and the leaf thickness is reduced in respect to control plants (a). Instead, Sculpure leaf fragments collected from control plants (b) do not apparently differ from fragments of ozonate plants (d,f) with the exception of stomata that appear mostly closed in the latter (d,e; compare with an open stoma, shown in b, of a control plant from OTCs CF).

Ultrastructural analysis by TEM of ozone-exposed Colombo plants confirmed that apparently undamaged cells, showing normal ultrastructure of membranes and organelles, were immediately adjacent to groups of plasmolysed cells around the substomatal cavity (**Figures 4.4a-d**). In these cells membranes were disrupted and chloroplasts swollen or burst, with remnants of grana dispersed in the cell (**Figures 4.4a,b,d**). Most of stomata, apparently open, showed only slight damages, with initial plasmolysis of guard and accessory cells (**Figure 4.4a**). A very different ultrastructural pattern was observed in Scultpur leaves exposed to ozone where almost all mesophyll and epidermal cells were unaltered with the exception of chloroplasts that appeared slightly swollen in respect to those of control plants grown in OTCs CF (**Figure 4.5a-c**). Also thylakoid structure was sometimes slightly swollen and numerous plastoglobules were present in the stroma, as a result of some physiological alteration, very likely an oxidative stress (**Figure 4.5c**).

Another peculiar feature of these plants was the presence of large and numerous starch grains in chloroplasts of guard cells of stomata (**Figure 4.5b**), which were mostly closed.

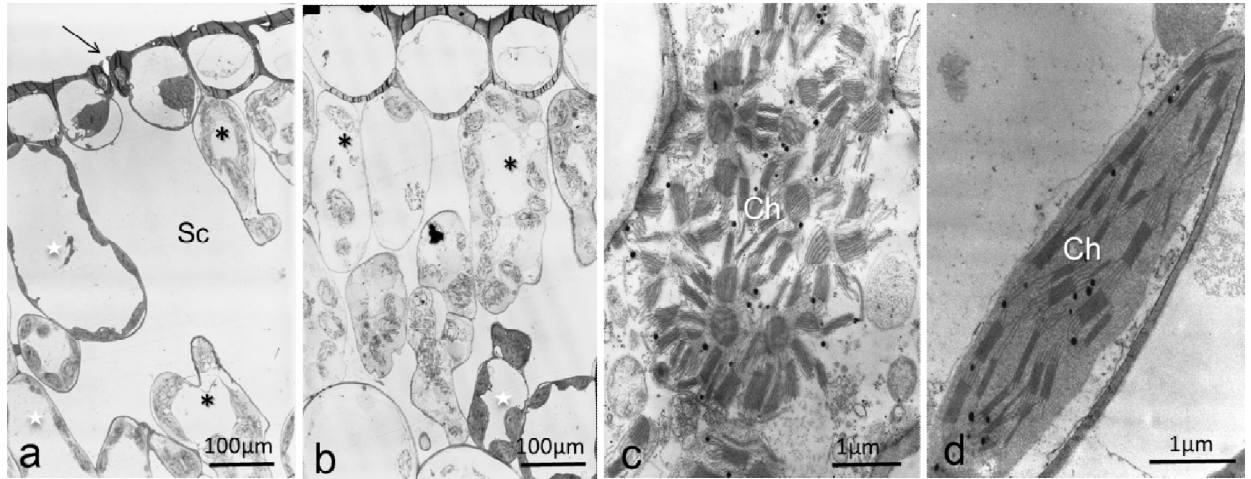


Figure 4. 4- Ultrathin cross sections of flag leaf of durum wheat, cv Colombo grown in OTCs OZ++ . In presence of chlorotic symptoms on May 27th , numerous mesophyll cells are heavily plasmolysed or dead (a and b, asterisks), particularly those close to the substomatal cavity (a, Sc). Stomata (arrow) appear mainly open (a). In plasmolysed cells chloroplasts (Ch) are very swollen or completely burst (c). In the adjacent mesophyll cells not plasmolysed (stars), cell membranes, chloroplasts and other organelles appeared unaltered (d).

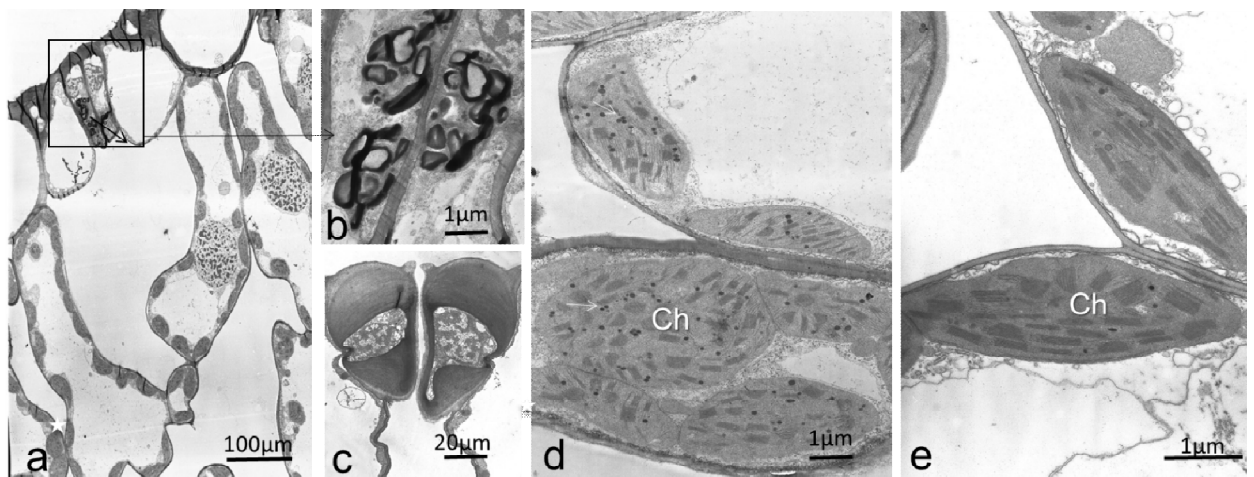


Figure 4. 5- Ultrathin cross sections of flag leaf of durum wheat cv Sculptur grown in OTCs OZ++ (a-d) or OTCs CF (e). On May 27th 2014, when leaves were still completely green, no plasmolysed cells were found in the mesophyll of ozonate plants (a). Stomata were often closed and guard cells contained large starch granules in chloroplasts (framed area in a, enlarged in b). Stomata closure was checked by serial sections up to the central part of stomatal aperture (c). Chloroplasts in all mesophyll cells appear almost unaltered, except for a slight swelling of the organelle and the thylakoid membranes (Ch) in respect to those observed in control plants from OTCs CF (e). Furthermore, chloroplasts from ozonate plants contained numerous plastoglobules (d, arrows).

4.4 Discussion

The purpose of this study was to shed light on the different symptomatic response of two durum wheat cultivars to ozone pollution and, in particular to understand why cv Sculptur, that shows yield losses comparable to cv Colombo under the same pollutant level, remains almost symptomless in comparison to the latter, which instead develops chlorotic and necrotic symptoms.

Biochemical analyses indicated that the content of the principal antioxidant metabolite, i.e. ascorbate, was significantly different in the two cultivars. In fact, the ascorbate pool was constitutively higher in Sculptur compared to Colombo, indicating a lower biosynthesis capacity of the latter. The non-significant differences between CF and OZ++ plants for AsA levels and AsA/(AsA + DHA) ratio may be interpreted as the existence of different pathways which ensure AsA recycling. This finding was observed in Sculptur in both sampling date, indicating that this cultivar is able to maintain the cellular ascorbate redox state. As regards to the glutathione content, our results indicated that up to the 14th May cv Colombo underwent a decrease in the glutathione redox state, as demonstrated by the increase in the glutathione oxidized pool. This finding suggests that at that first sampling data Colombo experienced a higher oxidative pressure, that in turn led to a lower capacity to regenerate the reduced glutathione pool. This result could be likely attributable to a lower activity of the glutathione reductase, the enzyme which catalyses the NADPH-dependent reduction of the disulphide bond of oxidized glutathione, and may be related to the lower AsA level which characterized Colombo compared to Sculptur. Conversely, at the sampling date the reduced glutathione pool and the GSH/GSH + GSSG ratio increased in both cultivars. Stimulation of GSH biosynthesis is a frequent response to stress condition and could suggest a plant attempt to compensate for decreases in the activity of enzymatic antioxidants, for example catalases (Noctor *et al.*, 1998).

Taken all together, these data indicate a better capacity of Sculptur to scavenge ROS production, particularly in the first weeks of ozone exposition, and explain the lack of visible symptoms on leaves until the beginning of senescence, which was however slightly anticipated in respect to control plants.

The ability of Sculptur to maintain redox homeostasis has been confirmed by microscopic observations. In fact, at the first sampling date, as soon as AOT40 overcame the critical threshold for herbaceous cultures, no H₂O₂ was detected in leaf tissues, contrary to Colombo in which H₂O₂ deposition was already high and some mesophyll cells

surrounding stomatal cavity were dead. This difference was present also at the second sampling date on May 27th, though at this time lapse some H₂O₂ deposits and dead cells were present also in the mesophyll of Sculptur. However, the number of dead or damaged cells was not sufficient to induce visible symptoms as in Colombo that already showed necrotic lesions at this stage. Histological and ultrastructural observations confirmed that damages due to oxidative stress in Colombo were mostly localized in the mesophyll close to substomatal cavity, very likely where the pollutant first impacts with leaf tissue. Nevertheless, groups of damaged cells in different stage of alteration were intermingled with apparent undamaged ones. This means that the level of ROS had overcome the lethal threshold in a patchy form over the leaf mesophyll, thus explaining the visible chlorotic and necrotic spots (Faoro and Iriti, 2009). The scattered distribution of damaged cells are unlikely due to different levels of ROS scavengers over the tissue. Instead, they could be the result of a higher raising of ROS concentration in some cells and not in others, as a consequence of another synergistic oxidative stress. A possible additive stress that raise ROS level in a similar patchy form is UV irradiation (Iriti *et al.*, 2007), thus is likely that light irradiation in very sensitive species, such as Colombo, may contribute to the ozone symptomatic response, or even induce by themselves alone visible symptoms. This could explain the chlorotic spots that appeared in Colombo grown in OTCs CF in the end of May, when the AOT40 in those OTCs was quite far from the critical threshold for ozone injury.

Histology of Sculptur leaves exposed to ozone was not appreciably different from that of control plant and no plasmolysis or other cell damages could be observed at light microscope level, except for the presence of a higher number of closed stomata, a well-known phenomenon induced by the pollutant in many plant species (Vahisalu *et al.*, 2010; Hayes *et al.*, 2015). However, when observed by TEM, Sculptur leaves grown in OTCs OZ++ showed some alterations of chloroplasts, present all over the mesophyll. These alterations regarded mostly the presence in the stroma of more numerous plastoglobuli in respect to control plants and a slight swelling of the organelles, typical of a mild oxidative stress (Violini *et al.*, 1992; Piller *et al.*, 2012;). All the other cell ultrastructures, including plasma membrane and tonoplast, appeared apparently undamaged. Interestingly, all the closed stomata, though apparently undamaged as well, contained numerous and large starch globules in their chloroplasts, that could partly account for their closure. In fact, it has been hypothesize that ROS generation in guard cells following an oxidative stress

leads to the inactivation of starch removal thus contributing to the stomata closure in an additional way (Leshem and Levine, 2013).

In the light of the above observations it can be concluded that Sculptur is more tolerant to ozone oxidative stress for the higher content of constitutive ascorbate pool that scavenges ROS more efficiently than in Colombo and for the synergistic effect of stomata closure that in Sculptur, not only occurs as the AOT40 overcome the critical threshold of 3000 ppb.h (see chapter 3) but seems to be maintained for a long time by the feed-back mechanisms of starch accumulation. This persistent stomata closure and the slight damages of chloroplasts account for the yield loss of Sculptur that was 13,0%, as average of two year experiments. Instead, the very similar yield loss of Colombo (-13,5%) are very likely attributable to the lower ROS scavenging capacity and to the less efficient stomata closure that led to severe damages of many mesophyll cells with the reduction of the active photosynthetic tissue by about 17%, as calculated by analyzing visible lesions on the leaf surface (see chapter 3).

Whatever the mechanisms underlying the loss of productivity caused by ozone in wheat it is clear that this plant species is very sensitive to oxidative stress by the pollutant, even when visible symptoms are not expressed and plants remain green almost up to senescence. Unfortunately, among the numerous cultivars of durum wheat so far analyzed (Picchi *et al.*, 2010; Monga *et al.*, 2015) those that do not show visible symptoms are really few and this poses another serious problem. In fact, chlorotic and necrotic symptoms induced by ozone are often mistaken for pathogen attack and this leads to unnecessary attempts at containment by applications of agrochemicals and consequent increase of costs and environmental impact, besides the danger of selecting pathogen resistant strains.

It is then highly desirable that geneticists in selecting cultivars of durum wheat pay more attention to those able to overcome more easily oxidative stress, keeping well in mind that breeding for high productive cultivars carried out in the last decades has led to the selection of photosynthetically very efficient plants with high stomatal conductance that inevitably uptake higher amount of ozone than old cultivars and are therefore more prone to ozone injury. Yield losses around 10-15% due to ozone pollution as many authors have reported, and we have confirmed in a two year experiments (see chapters 2 and 3), are indeed a significant amount. Considering also that in case the temperature of the planet by 2050 would increase as little as two degrees, the production of durum wheat would fall by about 20% (Amell *et al.*, 2013), this poses very serious food safety problems and any

measure to mitigate these losses, including the selection of ozone tolerant cultivars must be pursued soon.

Chapter 5

Ozone exposure and phytotoxic dose-response relationships for two durum wheat cultivars

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Abstract

The aim of this study was to identify the critical level for the protection of *Triticum durum* to ozone in Mediterranean conditions. Regression analysis here were performed using the ozone exposure-response and the phytotoxic stomatal dose-response relationships. The seasonal accumulation of phytotoxic stomatal ozone dose (POD₆) and of external ozone exposure (AOT40) were closely correlated with the reduction in the weight of grain yield, total aboveground biomass, stems, spikes and hectolitre weight. An average seasonal POD₆ of 10 mmol m⁻² corresponding approximately to an average seasonal AOT40 of 22071 ppb h induced a decline in the durum wheat grain production of about 18%. However, the accumulation of phytotoxic stomatal dose (POD₆) and hence, relative grain yield loss seem to be cultivar- and year-dependant. Year 2014 showed an increased POD₆ (-19%) compared to year 2013 resulting in greater grain yield loss for 2014 (-13%) compared to 2013. The AOT40 value was also slightly higher in 2014 (+6%) compared with 2013. Cv Colombo yielded higher POD₆ value (+6%) compared to cv Sculptur leading to remarkable reduction of grain yield in cv Colombo (-19%, cv Colombo versus -17% cv Sculptur). In general, our results indicate that ozone induced a 5% reduction in yield for each increment of 3.6 mmol m⁻²s⁻¹, POD₆ and 8300 ppb h, AOT40.

Results of this study demonstrate clearly that both relationships based on the ozone exposure and phytotoxic ozone dose proposed in the Mapping Manual could overestimate the ozone effects on durum wheat under Mediterranean conditions.

Key words: phytotoxic ozone dose; ozone exposure; critical levels; ozone concentration.

5.1 Introduction

Wheat is one of the world's most important cereals and counted among the staple food crops in the world (FAO, 2012; McFall & Fowler, 2009; Han *et al.*, 2015; Tibola *et al.*, 2016); McMullen., 2012). Although it is considered an ozone sensitive agricultural crop (Bagard *et al.*, 2015; Cao *et al.*, 2009; Feng *et al.*, 2011; Mills *et al.*, 2007), different species and cultivars exhibit a wide range of ozone sensitivity (Pleijel *et al.*, 2006; Gerosa *et al.*, 2014; Bagard *et al.*, 2015; Monga *et al.*, 2015). It has been observed that bread wheat (*Triticum aestivum*) is more O₃-sensitive than durum wheat (*Triticum durum*), but this could be influenced by parameters such as cultivar, cropping practices, geographic region and meteorological conditions (Reichenauer *et al.*, 1998; Herbiner *et al.*, 2002). Crop sensitivity to O₃ is typically assessed by evaluating the decline in plant growth and

crop yield and/or the appearance of O₃ injury (Evans, 1996; Karlsson *et al.*, 2004; Faoro and Iriti, 2005; Biswas *et al.*, 2008a; Gerosa *et al.*, 2009). Researchers agree on the fact that ozone-induced phytotoxic effects such as foliar injury, premature senescence, decreased growth and decline in yield are more closely related to the cumulative ozone uptake absorbed by the leaves than to the simple ambient ozone exposure (Fuhrer and Achermann, 1999; Massman *et al.*, 2000; Wieser *et al.*, 2000). Ozone negative effects on human health and ecosystems vegetation in Europe has been a concern for the United Nations Economic Commission for Europe (UNECE) and the European Union (EU) that led to the organization of the Convention on Long-Range Transboundary Air Pollution (LRTAP) for developing policies aimed at reducing ambient ozone concentrations. Such policies represent one of the objectives of the LRTAP firstly established in Geneva in 1979 by forty countries mostly European, which commits countries to cooperate for data collection and scientific information for the activation of specific strategies to control atmospheric emissions of pollutants. The risk assessment methods for ozone to estimate effects on vegetation used by the LRTAP Convention are based on the exceedances of ozone critical levels. These critical levels can be defined as the concentration, cumulative exposure (AOT40, accumulated ozone over a threshold of 40 ppb) or cumulative stomatal flux (e.g. AF_{st,y} or POD_y) above which direct adverse effects on sensitive vegetation may occur according to present knowledge” (LRTAP Convention, 2010). Critical level values based on exposure-response relationship are mainly derived from Open-Top Chambers experiments and do not take into account the stomatal influence on the amount of ozone entering the plant. The use of AOT40 index was agreed in 1996 at workshop in Kuopio (Finland) where the critical level values for crops, forest trees and semi-natural vegetation were established (Kärenlampi and Skärby, 1996). The common value of AOT40 that should not be exceeded for protecting agricultural crops and semi-natural vegetation from ozone damages, is 3000 ppb.h and is calculated as the sum of hourly ozone concentrations above a threshold of 40 ppb during daylight hours over a period of 3 month (May, June and July), when global radiation exceeds 50 W m⁻².

Evidences from several studies demonstrated that the models based on the calculation of stomatal ozone uptake seems to be more reliable in assessing the impacts of ozone on vegetation than the exposure-based models, because they take into account the environmental and phenological factors affecting the stomatal behavior and the consequent ozone dose absorbed (Danielsson *et al.*, 2013; Pleijel *et al.*, 2007). This approach require

the quantification of the phytotoxic ozone dose over a threshold Y , which represent the maximum value of instantaneous ozone stomatal flux detoxified by plant's defense systems, and the modeling of stomatal conductance. For wheat, a threshold of $6 \text{ nmol m}^{-2} \text{ s}^{-1}$ was proposed according to several experiments conducted in controlled conditions (Pleijel *et al.*, 2002; Danielsson *et al.*, 2003; LRTAP Convention, 2010). Mediterranean countries complain about the fact that models for assessing the ozone risk expressed such as phytotoxic dose of ozone (POD) are mainly based on the studies conducted in Central and Northern European environmental conditions (UNECE, 2010). And these models are suspected to overestimate the effects of this pollutant on crops and natural vegetation Southern Europe, where the climatic conditions are strongly different due to higher temperature and vapour pressure deficit (VPD) conditions, lower rain regimes and severe summer drought episodes (Emberson *et al.*, 2000; Mediavilla and Escudero, 2004; González-Fernández *et al.*, 2013).

The objective of this study is to identify dose-response relationships for *Triticum durum* yield losses based on AOT40 and POD₆ in order to evaluate their performance. These relationships will represent a contribution to local and large scale models currently used to estimate the ozone damages to vegetation at territorial level.

5.2 Materials and methods

5.2.1 Experiments analyzed

In this part, we analyzed the results from our two year experiments: an experiment on a varietal screening performed in 2013 (see chapter 2) and an experiment on the investigation of protective efficiency of chitosan from ozone performed in 2014 (see chapter 3). The first experiment investigated 5 cultivars of *Triticum durum* which were grown in 4 OTCs divided in two treatments (CF: ambient air concentration reduced at 50% and OZ++: ambient air concentration implemented with 50%), and each treatment was replicated in 2 OTCs. The second experiment analyzed two *Triticum durum* cultivars within 12 OTCs divided into 4 treatments (CF: filtered at -50 compared to ambient concentration, NF: non filtered, OZ+: ambient concentration incremented by 30 % and OZ++: ambient concentration incremented by 60%), and a sub-treatment with chitosan at 3 levels (CTRL: tap water, CHI40: chitosan with 40 kDa molecular weight and CHI300: chitosan with 300 kDa molecular weight). More details on these two experiments, can be found in the chapters 2 (2013) and 3 (2014). We extrapolated only the data relative to the

two commune cultivars for both experiments (Colombo and Sculptur). All the data available for the 2013 experiment were used for analysis, but for the 2014 experiment, we used only the data from the plants CTRL (e.i. plants not treated with chitosan). The ozone concentration has been monitored continuously at 1 meter height using an ozone analyzer (model 1108 RS, Dasibi Italia s.r.l., I) through a solenoid valve system that provided the switching from one to other OTC. Agro-meteorological parameters such as PAR (photosynthetically active radiation), air temperature, rainfall and relative humidity were also measured within the OTCs.

5.2.2 Calculation of accumulated ozone exposure (AOT40)

The accumulated ozone exposure over a threshold of 40 ppb hour (AOT40) was calculated by summing the hourly mean ozone concentration above 40ppb (for solar radiation exceeding 50 W.m^{-2}) and accumulated over the time. Detailed procedure for calculating the AOT40 is reported in the chapter 2 of this thesis.

(1)

$$AOT40 = \sum_{\substack{\forall [O_3] > 40 \text{ ppb} \\ \forall Rad \geq 50 \text{ W/m}^2}} ([O_3] - 40) \cdot \Delta t$$

5.2.3 Stomatal Conductance modelling and PODy calculation

The stomatal ozone dose received by plants was calculated by applying a multiplicative model for stomatal conductance (Jarvis, 1976) and a big-leaf ozone deposition scheme, following the methodology developed within the CLRTAP and described in the Mapping Manual (UNECE, 2008).

The hourly stomatal conductances to water (g_w) were calculated according to the following equation:

$$(2) \quad g_w = g_{w_{max}} * f_{phen} * f_{light} * \max\{f_{min}, (f_{temp} * f_{VPD} * f_{SWC})\}$$

where g_w is the actual stomatal conductance ($\text{mmol m}^{-2} \text{ PLA s}^{-1}$) and $g_{w_{max}}$ is the species-specific maximum stomatal conductance to water ($\text{mmol H}_2\text{O m}^{-2} \text{ PLA s}^{-1}$). The f functions (all ranging between 0 and 1) describe the relative effects of the phenology and of the different environmental conditions (temperature, Vapour pressure deficit VPD, soil water content SWC) on $g_{w_{max}}$, to calculate the relative stomatal conductance g_w . The f_{min} represents the minimum stomatal conductance expressed relatively to $g_{w_{max}}$.

The $g_{w,max}$ value for *Triticum durum* was assumed to 618 mmol m⁻² PLA s⁻¹ as reported by González-Fernández *et al.* (2013) in a similar work conducted under Mediterranean conditions.

The phenology function f_{phen} was based on the effective temperature sum accumulation tt (°C) according to the equations (3, 4, 5). A_{start} and A_{end} are the temperature sums of the start and the end of the dose accumulation period respectively. The parameters f_{phen_a} and f_{phen_b} denote the maximum fraction of $g_{w,max}$ that g_w takes at the start and end of the accumulation period for ozone flux. f_{phen_c} to f_{phen_i} are wheat receptor-specific parameters describing the shape of the function within the accumulation period.

$$(3) \text{ when } A_{start} \leq tt < (A_{start} + f_{phen_e}), \quad f_{phen} = 1 - \left(\frac{1-f_{phen_a}}{f_{phen_e}} \right) ((A_{start} + f_{phen_e}) - tt)$$

$$(4) \text{ when } (A_{start} + f_{phen_e}) \leq tt \leq (A_{end} - f_{phen_f}), \quad f_{phen} = 1$$

$$(5) \text{ when } (A_{end} - f_{phen_d}) < tt \leq A_{end}, \quad f_{phen} = 1 - \left(\frac{1-f_{phen_b}}{f_{phen_f}} \right) (tt - (A_{end} - f_{phen_f}))$$

tt was calculated using a base temperature of 0°C day at the anthesis date, and in the present study tt ranged between -300°C day to 675°C day according to the wheat parameterization of González-Fernández *et al.* (2013). These values corresponded to the period between 10th May and 30th June 2013 (about 3 weeks before and 4.4 weeks after the average date of the anthesis) for the cultivar cv Sculptur and from 13th May to 02nd July 2013 (about 3 weeks before and four 4.3 weeks after the average date of anthesis) for the cultivar cv Colombo. In 2014 the accumulation period ranged between 28th April to 18th June (about 3 weeks before and 4.6 weeks after the average date of anthesis) for cv Sculptur and between 02 May to 20th June (about 3 weeks before and 4.4 weeks after the average date of anthesis) for cv Colombo.

The f_{light} function that was

$$(6) \quad f_{light} = 1 - \exp((-light_a) * PFD)$$

where PFD represents the photosynthetic photon flux density in units of $\mu\text{mol m}^{-2} \text{s}^{-1}$ and $light_a$ is a shaping parameter indicated in Table 5.1.

The function used to describe f_{temp} is given in the Equation (7, 8):

$$(7) \text{ when } T_{min} < T < T_{max}, \quad f_{temp} = \max \{ f_{min}, [(T - T_{min}) / (T_{opt} - T_{min})] * [(T_{max} - T) / (T_{max} - T_{opt})]^{bt} \}$$

$$(8) \text{ when } T_{min} > T > T_{max}, \quad f_{temp} = f_{min}$$

where T is the air temperature in $^{\circ}\text{C}$, T_{min} and T_{max} are the minimum and maximum temperatures at which stomatal closure occurs, T_{opt} is the optimum temperature and bt is a shaping parameter defined as follows:

$$(9) \quad bt = (T_{max} - T_{opt}) / (T_{opt} - T_{min})$$

The adopted values of T_{max} , T_{min} and T_{opt} are indicated in Table 5.1. The response of the stomata to the air drying potential VPD is described by the f_{VPD} function as follows (Equation (10))

$$(10) \quad f_{VPD} = \min\{1, \max\{f_{min}, ((1-f_{min})*(VPD_{min} - VPD) / (VPD_{min} - VPD_{max})) + f_{min}\}\}$$

where VPD is the actual vapour pressure deficit (KPa) and VPD_{max} is the VPD value above which the stomata begins to close under dry conditions, and VPD_{min} is the VPD values at which the stomatal reached their minimum aperture ($f_{VPD}=f_{min}$). For this specific application the values indicated by González-Fernández *et al.* (2013) were used, i.e. VPD_{max} of 3.1 kPa and VPD_{min} of 4.8 kPa. In this study f_{SWC} was not considered and set equal to 1 because plants were always kept well watered. All the parameters used in this application have been taken from the model presented by González-Fernández *et al.* (2013) and summarized in Table 5.1.

Once g_w was obtained, the phytotoxic ozone dose POD was calculated from the ozone concentration measured inside the OTCs by means of the big-leaf resistive scheme described in the Mapping Manual.

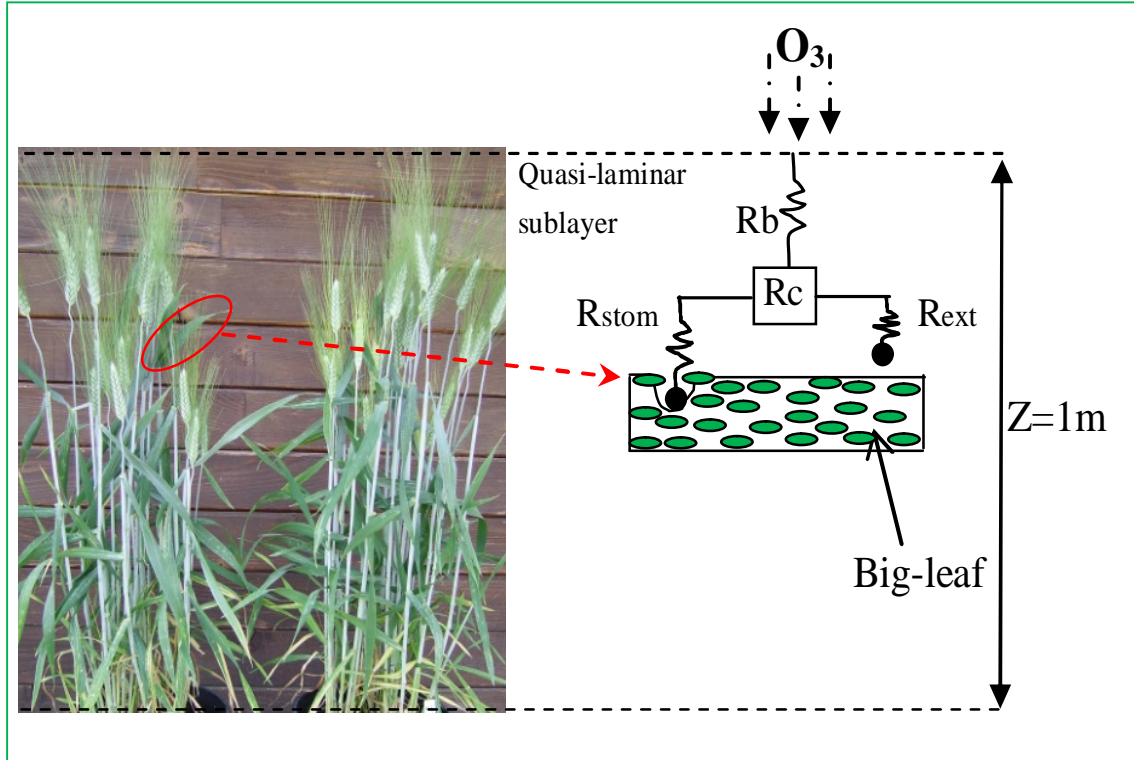


Figure 5. 1- Resistance analogy for the dry deposition of atmospheric pollutants, “big-leaf” Model.

This scheme (**Figure 5.1**) includes also the ozone deposition on the external leaf surfaces (cuticles) represented by a resistance to ozone deposition R_{ext} of 2500 s/m (UNECE, 2010) per unit of vegetal surface ($SAI = \text{green} + \text{senescent LAI}$).

Synthetically:

$$(11) \quad F_{\text{stom}, O_3} = O_3 \cdot (LAI \cdot g_w \cdot 0.663) \cdot [R_c / (R_b + R_c)]$$

where R_c is the canopy resistance to the ozone deposition, resembling both the ozone uptake by stomata and the ozone deposition on the external cuticles.

$$(12) \quad R_c = 1 / (g_w \cdot 0.663 \cdot LAI + SAI / R_{ext}) \quad [s \cdot m^{-1}]$$

and R_b is the resistance which ozone experiences when it crosses the sub-laminar layer.

$$(13) \quad R_b = 1.3 \cdot 150 \cdot \sqrt{d/u} \quad (\text{McNaughton and van der Hank, 1995})$$

where u is the wind speed inside the OTCs and d is the crosswind leaf dimension, which was set to an average value of 2 cm, according to the measurements of the leaves dimensions.

The 0.663 value in the above equations represents the diffusivity ratio between O_3 and water vapour in air (Massmann, 1998; Nobel, 1999) which is needed to convert the stomatal conductance to water in stomatal conductance to ozone. The 1.3 and 150 values are the empirical values given by McNaughton and van der Hank (1995) for their R_b

parameterization. In this work a unitary LAI and SAI was considered for all the calculations.

Finally, the phytotoxic ozone dose (POD_y) was calculated by integrating the hourly ozone stomatal uptake $F_{\text{stom}, \text{O}_3}$ (in $\text{nmol m}^{-2} \text{ PLA s}^{-1}$) along the growing season with the application of the detoxifying threshold Y of $6 \text{ nmol m}^{-2} \text{ s}^{-1}$ provisionally suggested by the Mapping Manual.

$$(14) \quad \text{POD}_Y = \sum \left[\left(F_{\text{stom}, \text{O}_3} - Y(\text{nmol m}^{-2} \text{ s}^{-1}) \right) * 3600(\text{s h}^{-1}) * 10^{-6} \right]$$

Table 5. 1- Parameterization of the multiplicative model to estimate gw for durum wheat. f_{\min} is the fraction of $g_{w_{\max}}$ at minimum gw; A_{start} and A_{end} represent the start and the end of the accumulation period in degree-days relative to anthesis. a and b are the difference between the maximum $g_{w_{\max}}$ fraction (2) and the f_{phen} value at the start of flag-leaf senescence and A_{end} respectively; fraction of $g_{w_{\max}}$; e, f, g, h, i , are the temperature sums at A_{start} , anthesis, end of maximum gw, start of flag-leaf senescence and temperature sum at which f_{\min} is reached; a is the rate of saturation of gw in response to photosynthetic active radiation; T_{\min} and T_{\max} denote the temperatures below and above gw is limited to f_{\min} ; T_{opt} is non-limiting temperature for gw; VPD_{\max} and VPD_{\min} define the level when vapour pressure deficit starts to limit gw and f_{\min} are reached respectively; SWC_{\max} and SWC_{\min} are the minimum non-limiting soil water content and the value below which gw is limited to f_{\min} respectively.

Function	Parameters	Units	Value
$g_{w_{\max}}$		$\text{mmol H}_2\text{O m}^{-2} \text{ PLA s}^{-1}$	618
f_{\min}		<i>fraction</i>	0.01
f_{phen}	A_{start}	$^{\circ}\text{C.day}$	300
	A_{end}	$^{\circ}\text{C.day}$	675
	a	<i>fraction</i>	1
	b	<i>fraction</i>	0
	c	$^{\circ}\text{C. day}$	0
	d	$^{\circ}\text{C. day}$	0
	e	$^{\circ}\text{C. day}$	300
	f	$^{\circ}\text{C. day}$	0
	g	$^{\circ}\text{C. day}$	100
	h	$^{\circ}\text{C. day}$	675
	i	$^{\circ}\text{C. day}$	675
f_{light}	a	<i>Constant</i>	0.0105
f_{temp}	T_{\min}	$^{\circ}\text{C}$	11
	T_{opt}	$^{\circ}\text{C}$	28
	T_{\max}	$^{\circ}\text{C}$	45
	bt	<i>fraction</i>	1
f_{VPD}	VPD_{\max}	kPa	3.1
	VPD_{\min}	kPa	4.9
f_{SWC}	SWC_{\max}	%	-
	SWC_{\min}	%	-

5.2.4 Data treatment and statistical analysis

The statistical unit per regression analysis was the OTC, and therefore the yield parameters for each OTC have been obtained averaging the values of each plant within the OTC, per each cultivar. The yield parameters of each experiment have been normalized by calculating the relative yield (RY). This latter has been obtained dividing the yield observed in each treatment for the yield at exposure or at zero dose (RY_0), in turn obtained as the intercept of the yields regression of each experiment versus the exposure (AOT40) and the phytotoxic stomatal dose (POD_6). The exposure-effects and dose-effects relationships have been obtained assembling the results of two year experiments end using as relative yields the mean of relative yield of all the OTCs with same treatment, and as dose and exposure the means of the exposure and of the dose of all the OTCs with same treatment. This is the procedure commonly followed within the framework of ICP Vegetation (UN/ECE) when results of different experiments are compared for identifying the dose-effect relationships (Fuhrer *et al.*, 1997; Pleijel, 2011; González-Fernández *et al.*, 2013; 2014). All the analysis have been performed using the STATISTICA v.8.0 software (Tulsa, USA).

5.3 Results and discussion

5.3.1 AOT40, POD_6 and their relative effects

In both the 2013 and 2014 experiments the ozone exposure in the control treatments CF was well below the AOT40 critical level set by UN/ECE for crop protection (5 times in 2013 and 3 times in 2014), and by contrast it was more than 7 times above the critical level in the most ozonated treatments for both years (**Table 5.2**).

The POD_6 value in the control treatment for both years was 5 times below the critical dose suggested by the Mapping Manual (1 mmol m^{-2} for grain yield) (LRTAP, 2004) and, by contrast, in the most ozonated treatment (OZ++) it was roughly 9 times above the critical level in 2013 and 10 to 11 times in 2014. The seasonal POD_6 for plants within the NF and OZ+ treatments was 3.1 and 5.4 mmol m^{-2} respectively. The latter results are in agreement with the findings of Grünhage *et al* (2012) who reported the annual POD_6 value between 2 and 5 mmol m^{-2} PLA for bread wheat grown in non limited soil water content conditions (SWC).

In both years the cv Colombo absorbed a slightly higher phytotoxic ozone dose than cv Sculptur showing about 3% and 8% more in 2013 and 2014, respectively, in the OZ++ treatment.

The relative effects of the cumulative ozone exposure (AOT40) and of the phytotoxical stomatal dose (POD₆) on grain yield, aboveground biomass, spikes, stems and hectolitre weight under the different ozone treatments in the two experiments are illustrated in **Table 5.2**. The relative effect was calculated as the ratio of the effect of each ozone treatment to the effect observed in the charcoal filtered OTCs (i.e. relative yield=100%). The values indicated in **Table 5.2** are the average of the relative yield of all the OTCs with the same ozone treatment.

All yield parameters were negatively affected in cv Colombo by both ozone exposure and dose in all the ozone treatments both years, with the only exception of the grain weight and stems weight of the NF treatment in 2014. Also cv Sculpur resulted negatively affected by ozone in all yield parameters but only in the most ozonated treatment (OZ++). In the intermediate treatments (NF and OZ+), despite the observed exceedance of the critical level and dose, no effect was found on almost the yield parameters except for the slight decrease recorded in the stems weight.

Table 5. 2 - Seasonal mean value of ozone exposure (expressed as AOT40, unit: ppb h) and phytotoxic ozone dose (POD₆, mmol m⁻²) correlated with average relative yield of the grain, aboveground biomass, spikes, stems and hectolitre weight for durum wheat plants grown under different ozone concentration levels in the 2013 and 2014 experiment. “-” indicates that in the 2013 experiment this ozone level was not applied (no value); R. effect: relative effect. The ozone effect in this table was calculated based only on the plants within the OTCs (two replications per treatment) where ozone concentrations were monitored.

<i>Cultivar</i>	<i>COLOMBO</i>						<i>SCULPTUR</i>					
	<i>Year 2013</i>			<i>Year 2014</i>			<i>Year 2013</i>			<i>Year 2014</i>		
<i>O₃ levels</i>	<i>AOT40</i>	<i>POD₆</i>	<i>R. effect</i>	<i>AOT40</i>	<i>POD₆</i>	<i>R. effect</i>	<i>AOT40</i>	<i>POD₆</i>	<i>R. effect</i>	<i>AOT40</i>	<i>POD₆</i>	<i>R. effect</i>
<i>Relative Grain Yield</i>												
<i>CF</i>	682	0.2	100%	1120	0.2	100%	682	0.2	100%	1120	0.2	100%
<i>NF</i>	-	-	-	6580	3.4	102.3%	-	-	-	6580	3.1	103.1%
<i>OZ+</i>	-	-	-	9932	5.4	98.3%	-	-	-	9932	5.0	109.4%
<i>OZ++</i>	21423	9.2	90%	22718	11.2	72.9%	21423	8.9	84%	22718	10.4	81.4%
<i>Relative Aboveground Biomass</i>												
<i>CF</i>	682	0.2	100%	1120	0.2	100%	682	0.2	100%	1120	0.2	100%
<i>NF</i>	-	-	-	6580	3.4	96.2%	-	-	-	6580	3.1	107.3%
<i>OZ+</i>	-	-	-	9932	5.4	91.3%	-	-	-	9932	5.0	104.0%
<i>OZ++</i>	21423	9.2	76%	22718	11.2	75.2%	21423	8.9	80%	22718	10.4	88.5%
<i>Relative Stems Weight</i>												
<i>CF</i>	682	0.2	100%	1120	0.2	100%	682	0.2	100%	1120	0.2	100%
<i>NF</i>	-	-	-	6580	3.4	102.3%	-	-	-	6580	3.1	117.8%
<i>OZ+</i>	-	-	-	9932	5.4	90.1%	-	-	-	9932	5.0	97.6%
<i>OZ++</i>	21423	9.2	68%	22718	11.2	82.9%	21423	8.9	78%	22718	10.4	96.7%
<i>Relative Spikes Weight</i>												
<i>CF</i>	682	0.2	100%	1120	0.2	100%	682	0.2	100%	1120	0.2	100%
<i>NF</i>	-	-	-	6580	3.4	92.6%	-	-	-	6580	3.1	101.7%
<i>OZ+</i>	-	-	-	9932	5.4	92.0%	-	-	-	9932	5.0	107.4%
<i>OZ++</i>	21423	9.2	83%	22718	11.2	70.6%	21423	8.9	82%	22718	10.4	84.2%
<i>Relative Hectolitre Weight</i>												
<i>CF</i>	682	0.2	100%	1120	0.2	100%	682	0.2	100%	1120	0.2	100%
<i>NF</i>	-	-	-	6580	3.4	101%	-	-	-	6580	3.1	100%
<i>OZ+</i>	-	-	-	9932	5.4	97%	-	-	-	9932	5.0	100%
<i>OZ++</i>	21423	9.2	99%	22718	11.2	98%	21423	8.9	97%	22718	10.4	96%

5.3.2 Linear regression of yield parameters on AOT40 and POD₆

The **Table 5.3** shows the linear regression of yield parameters and statistical significance on ozone exposure and phytotoxic stomatal ozone dose for each *Triticum durum* cultivar. The linear regression related to the AOT40 resulted in a significant decrease of grain yield ($P=0.0480$), total aboveground biomass ($p=0.0271$) and spikes weight ($p=0.0189$) for cv Colombo. The only significant reduction caused by ozone exposure in the yield parameters for cv Sculptur was the hectolitre weight ($p=0.0080$). Based on the POD₆ relationships, the linear regression for cv Colombo was quasi-significant for grain yield ($p=0.0533$) and significant for aboveground biomass ($p=0.0409$) and spikes weight ($p=0.0245$). Again in the case of cv Sculptur a unique significant reduction was found in the hectolitre weight ($p=0.0107$). Although the R^2 values are relatively low, it appears clearly that the main yield parameters tend to be decreased at the increasing of exposure and phytotoxic stomatal dose. The trend in cv Colombo is a reduction in grain yield of 2.4 and 1.6 % for each increase of 3000 ppb.h of AOT40 and 1 mmol m⁻² of POD₆ respectively. While in cv Sculptur this reduction was 1.6 and 1% for each increase of 3000 ppb and 1 mmol m⁻². Analogous reductions were observed for the total aboveground biomass and spikes weight in cv Colombo but not in cv Sculptur. On the basis of the regression slopes, cv Colombo confirms to be relatively more ozone-sensitive than cv Sculptur.

Table 5. 3 - Regression analysis of the yield parameters versus the ozone exposure (AOT40) and the phytotoxical stomatal dose (POD6) for each *Triticum durum* cultivar. Each point represents the average value of the plants' yield within each OTC (three replications per treatment). The regression is considered significant for * $p \leq 0.05$, ** $p \leq 0.01$ and * $p \leq 0.001$.**

Relative yield	COLOMBO			SCULPTUR		
	R ²	Equation	p	R ²	Equation	p
Linear regression of yield parameters on AOT40						
Grain yield	0.2878	$y = -8E-06x + 0.9999$	0.0480	0.144	$y = -5E-06x + 0.9988$	0.1183
Abg. biomass	0.3452	$y = -8E-06x + 1.0059$	0.0271	0.1701	$y = -5E-06x + 1.0294$	0.3558
Hectolitre weight	0.1679	$y = -1E-06x + 0.9989$	0.1457	0.0504	$y = -1E-06x + 0.9921$	0.0080
Harvest index	0.0079	$y = 8E-07x + 0.9913$	0.7622	0.002	$y = -3E-07x + 0.9722$	0.1621
Stem weight	0.2093	$y = -7E-06x + 1.0105$	0.1000	0.0696	$y = -5E-06x + 1.0589$	0.9454
Spikes weight	0.3801	$y = -9E-06x + 1.0021$	0.0189	0.2094	$y = -6E-06x + 1.0137$	0.1423
Linear regression of yield parameters on POD6						
Grain yield	0.2767	$y = -0.0156x + 0.9994$	0.0533	0.1577	$y = -0.012x + 1.0015$	0.1679
Abg. biomass	0.3042	$y = -0.0162x + 1.0018$	0.0409	0.0478	$y = -0.0062x + 1.0038$	0.4781
Hectolitre weight	0.1906	$y = -0.0026x + 0.9998$	0.1185	0.4328	$y = -0.0041x + 0.9999$	0.0107
Harvest index	0.0012	$y = 0.0006x + 0.996$	0.9070	0.1686	$y = -0.006x + 0.9973$	0.1338
Stem weight	0.1687	$y = -0.0139x + 1.0036$	0.1447	0.0005	$y = 0.0009x + 1.0061$	0.9062
Spikes weight	0.3550	$y = -0.0173x + 1.0002$	0.0245	0.1359	$y = -0.0101x + 1.0026$	0.2081

5.3.3 Exposure-effect and dose-effect relationships

Both exposure-effect and phytotoxic dose-effect induced a statically significant reduction of grain yield and aboveground biomass for *Triticum durum* regardless of cultivar factor (i.e. for grain yield $p=0.0154$ and 0.0053 using the AOT40- and POD₆-based effect respectively).

In general the POD₆-based relationships presented the best fit compared to the AOT40-based relationships (e.g. based on relative grain yield, $R^2= 0.4596$ and 0.5572 using AOT40 and POD₆ respectively). The relationships between relative yield and POD₆ showed the higher R^2 values than for those between relative yield and AOT40 for grain yield and hectolitre weight. On the contrary the AOT40-based relationships yielded a higher R^2 compared to the POD₆.

Despite the diversity of responses from the two durum wheat cultivars, there is evident trend to a reduction in grain yield and total aboveground biomass at increasing of both the exposure and the phytotoxic stomatal dose. In general it has been observed for *Triticum durum* regardless of cultivar type a reduction in grain yield of (1.8%) for each increase of 3000 ppb h of AOT40. A reduction of 1.3% was found for each increase of 1 mmol m^{-2} of POD₆. The relatively low reductions was observed in the total aboveground biomass and hectolitre weight for both AOT40 and POD₆. The relationships between ozone effects and yield parameters reduction are reported in the **Figure 5.2**.

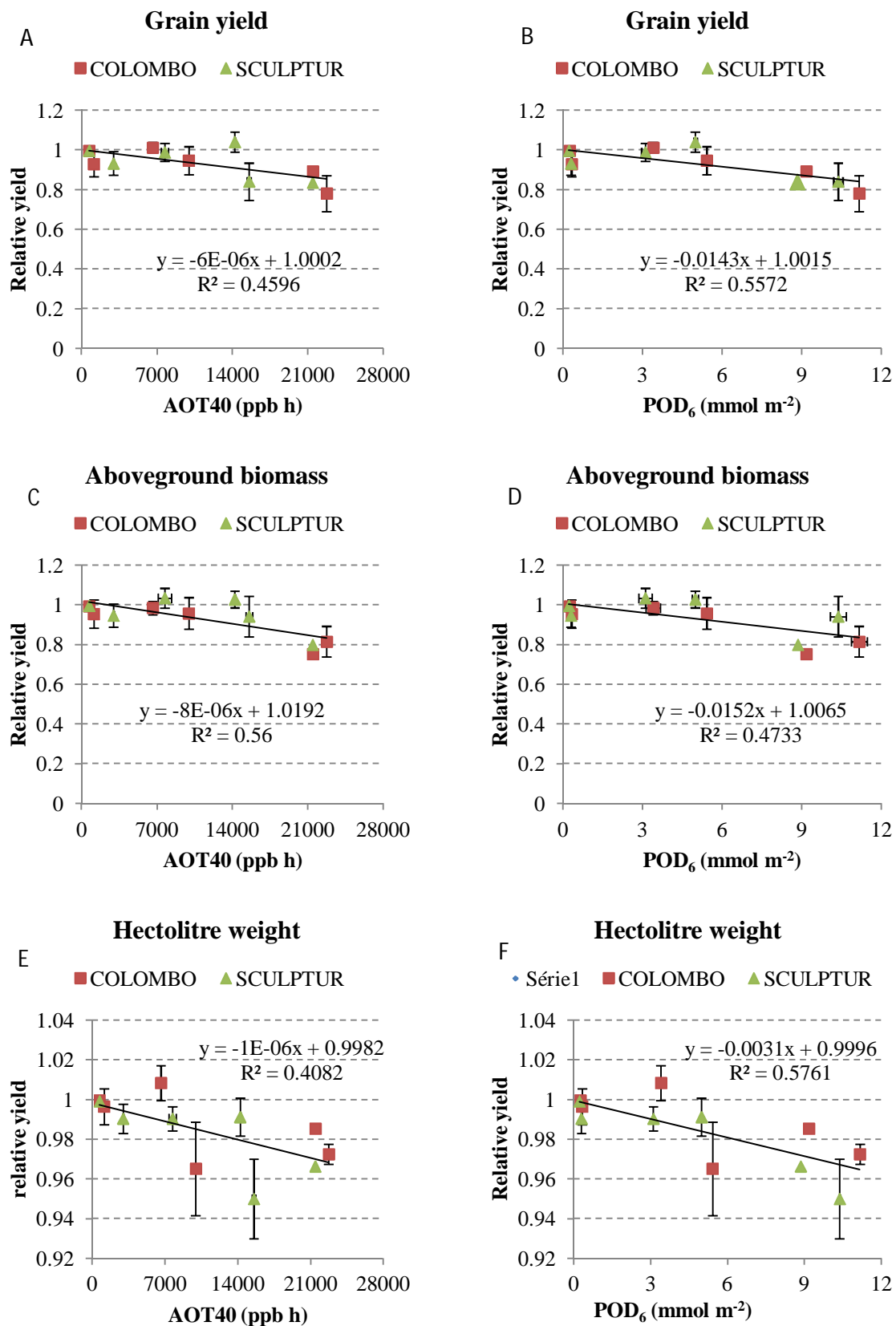


Figure 5. 2- The proposed exposure-effect and dose-effect relationships for *Triticum durum*. Left graphs refer to AOT40 and right graphs to POD₆ relationships. Vertical bars indicate standard error of means for relative yields and horizontal bars standard error for exposure and dose.

In this study the AOT40 value corresponding to a 5% reduction of grain yield was around 6200 ppb h for cv Colombo and 9600 ppb h for cv Scultur. These values are about 2-3 times greater than that established in the Mapping Manual and, remarkably, greatly vary between one cultivar and the other. Again these values are significantly higher than the critical AOT40 found by Gelang *et al.*, (2000) and Wang *et al.* (2012) who reported 3300 ppb h and 2000 ppb h respectively. From this evidence, we propose an AOT40-based critical level of 8000 ppb h for durum wheat protection. Our critical level might be more appropriate for an application in the Mediterranean countries where *Triticum durum* is decidedly more grown and consumed than the *Triticum aestivum*.

According to this study the POD₆ value causing a 5% of relative grain yield was around 3 mmol m⁻² for cv Colombo and 4 mmol m⁻² for cv Scultur. These critical doses are about 3-4 times higher than those indicated in the Mapping Manual for wheat detoxification (Pleijel, 1996). Grünhage *et al.* (2012) and Pleijel *et al.* (2007) have found a critical POD₆ dose of 1.3 mmol m⁻² for a 5% grain yield loss in *Triticum aestivum*. Feng *et al.*, 2012 suggested a critical POD₆ of 1 mmol m⁻² causing the same relative grain yield reduction for winter wheat grown in subtropical China. Comparing our results with those of Pleijel. (1996), Grünhage *et al.* (2012) and Pleijel *et al.* (2007) and Feng *et al.* (2012) reporting a POD₆ critical value around 1 mmol m⁻² for the 5% reduction of grain yield, we can confirm that *Triticum durum* is more ozone-tolerant than *Triticum aestivum*. However, it is important to emphasize that *Triticum durum* manifests also the significant ozone damages. Our results agree with the findings of Reichenauer *et al.* (1998), Herbinger *et al.* (2002) and Biswas *et al.* (2008a) suggesting that *Triticum aestivum* is more ozone-sensitive than *Triticum durum*. Present results confirm again the ozone-tolerance of durum wheat reported in our previous study (Gerosa *et al.*, 2014). On the basis of our results we propose a critical POD₆ of 3.5 mmol m⁻² for a 5% reduction of grain yield to be used in the Mediterranean countries for *Triticum durum*. Analogously the AOT40 critical level could be set to 8000 ppb h for *Triticum durum*. This imply that the current value of AOT40 (3000 ppb h) and POD₆ (1 mmol m⁻²) proposed in the Mapping Manual as critical level above which direct adverse effects may occur in wheat (Kärenlampi and Skärby, 1996) could remarkably overestimate the ozone damages in the durum wheat under south Europe conditions. To our knowledge this is the first attempt to derive a dose-effect relationship for *Triticum durum*.

Further investigations on ozone effect on durum wheat in the same region are needed for better definition of both exposure and dose based critical levels of ozone.

General conclusions

The experiments carried out in two growing seasons on the effects of the tropospheric ozone on *Triticum durum* in Open-Top Chambers facilities, allow to draw the following conclusions.

1. Durum wheat has confirmed to be an ozone sensitive crop species though with a strong variability among cultivars. At this regard the most productive cultivars (Colombo and Sculptur) proved to be particularly sensitive to the pollutant.
2. Colombo and Sculptur showed significant yield losses in two growing seasons, with an average of 13.3% and 13.0%, respectively, in terms of grain weight per plant. Nevertheless, their symptomatic response in terms of visible symptoms was very different, being Sculptur almost symptomless, even after several weeks of ozone exposition, contrary to Colombo that showed in a few weeks typical ozone-like symptoms (chlorotic spots, often degenerating in necrotic lesions).
3. Eco-physiological parameters revealed that the main event associated to ozone exposition was the great reduction of stomatal conductance in both cultivars, with particular reference to Sculptur that first showed this reduction, about ten days before Colombo. However, the reduction of stomatal conductance may account for yield loss but not for the different symptomatic response.
4. Biochemical and microscopic observations indicated that Sculptur is capable to maintain redox homeostasis better than Colombo, thus limiting ROS damages. This ability is certainly due to its higher basal level of reduced ascorbate but also to a possible avoidance effect as a consequence of a more rapid stomata closure. The latter has been confirmed also by microscopic analysis that showed an involvement of starch accumulation in stomatal guard cells, as a possible mechanism driving the phenomenon. In any case, the lower accumulation of ROS in Sculptur leaf tissues, whatever the reasons, only slightly altered cell ultrastructures thus explaining the lack of visible symptoms. In Colombo, instead, the lower ascorbate content and the possible delayed stomata closure caused ROS accumulation over a lethal threshold in groups of mesophyll cells around substomatal cavity, leading to the appearance of chlorotic/necrotic lesions.

5. The application of chitosan as leaf spraying showed contrasting effects in protecting plant from ozone injury. Positive effect of chitosan in terms of grain yield was observed only in the plants exposed to low ozone concentrations but not to elevated concentrations possibly because in the latter conditions the stomata closure transiently induced by this compound cannot influence significantly the uptake of the pollutant. However, in any case chitosan treatments influenced symptom expression.
6. Stomatal flux analysis showed that each increment of ozone exposure of 3000 ppb.h resulted in durum wheat yield reduction of 1.8%. Additionally, each accumulation of the phytotoxic stomatal ozone dose of $1 \text{ mmol m}^{-2} \text{ s}^{-1}$ caused 1.3% in yield loss. Both ozone exposure (as AOT40) and phytotoxic stomatal ozone dose (as POD₆) can be considered good approaches for ozone risk assessment in durum wheat in the Mediterranean conditions, though POD₆ shows a better fit compared to the AOT40. In particular, to protect durum wheat (*Triticum durum*) from ozone in this region, the use of the critical levels values based on ozone exposure and phytotoxic stomatal ozone dose of 8000 ppb h and $3.5 \text{ mmol m}^{-2} \text{ s}^{-1}$ respectively seem reliable from the data of this study.

As a general conclusion, this work showed that durum wheat cultivars showing O₃ foliar injuries (the ozone-like syndrome) do not always result in crop yield losses greater than symptomless ones, indicating that there is little or no correlation between visible O₃ damage and reduced yield. This fact suggests a revision of the O₃ ‘tolerance’ and ‘sensitivity’ definitions, as it is evident that the O₃ sensitivity shown by plants for a certain parameter (e.g. foliar symptoms) is not always related to other parameters (e.g., agronomic yield). Therefore, it is necessary to develop an integrated approach that considers all these aspects. In the case of durum wheat, where the economic aspects are more relevant than the ecological aspects, it seems more sound to base this definition on the agronomical yield/loss.

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